Study of inhibiting properties of *Trachyspermum ammi* on uric acid crystallization

Project report submitted to
Visvesvaraya National Institute of Technology, Nagpur
in partial fulfillment of the requirements for the award of
the degree

Master of Technology
in
Chemical Engineering

by

Akshay S. Dorlikar

Roll No. MT23MCL002

under the guidance of

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Nagpur 440 010 (India)

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I, Akshay S. Dorlikar, hereby declare that this dissertation titled **"Study of inhibiting properties of *Trachyspermum ammi* on uric acid crystallization"** is carried out by me in the Department of Chemical Engineering of Visvesvaraya National Institute of Technology, Nagpur. The work is original and has not been submitted earlier whole or in part for the award of any degree/diploma at this or any other Institution / University.

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ABSTRACT

Uric acid (UA) nephrolithiasis represents a growing clinical challenge, with current therapeutic options offering limited efficacy in preventing UA crystal formation. In this study, the antiurolithiatic potential of *Trachyspermum ammi* (Ajwain) seed extract was explored through a comprehensive in vitro analysis of its ability to inhibit UA crystallization. The aqueous extract was prepared via decoction and subjected to phytochemical screening, which revealed the presence of key bioactive compounds such as phenols, flavonoids, saponins, glycosides, and thymol. Total phenolic content (TPC), Total flavonoid content (TFC), and Total antioxidant capacity (TAC) were evaluated, confirming strong antioxidant properties that may contribute to the inhibition of UA nucleation and aggregation.

Crystallization experiments were conducted using synthetic urine and uric acid stock under controlled pH and temperature, and the effect of extract concentration and pH on induction time and crystal size was assessed. Results demonstrated a significant increase in induction time at pH 4, 4.5, 5 is 106, 30, 74 times increases and a marked reduction in crystal size with increasing extract concentration for pH 4, 4.5 by 6 times and 11 times for 5 pH, indicating dose-dependent inhibition of UA crystallization. Microscopic and spectroscopic analyses, including FTIR shows that *T. ammi* extract does not chemically modify uric acid but exert its inhibitory effect on crystal formation through mild, non-covalent interactions. PXRD shows that the extract, even at higher concentrations, does not cause a significant disruption to the crystallization, SEM and EDS, provided evidence of molecular interaction between the extract and UA crystals, resulting in structural distortion, reduced crystallinity, and altered morphology.

These finding suggests that *T. ammi* extract can effectively delay UA nucleation and crystal growth. This highlights its potential as a natural therapeutic agent for preventing uric acid kidney stones and warrants further investigation into its mechanism and clinical applicability.

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1. INTRODUCTION

1.1 Introduction to Kidney Stone

Kidney stones also known as renal calculi, are hard crystalline structures that form within the kidneys due to the supersaturation of urine along with stone forming salts and compounds[1]. These stones may pass out of the body through the urinary tract unnoticed when they are small. However, when they grow larger, typically 2 to 3 mm or more, they may obstruct the ureter, leading to a variety of clinical symptoms. These include severe colicky pain (renal colic), haematuria (blood in the urine), urine flow, urinary tract infections obstruction, and hydronephrosis, which is the swelling of one or both kidneys due to urine retention [2], [3]. The harbinger of pathogenesis of the kidney stone formation is not a simple one-time event but involves a convoluted interaction of physiological, dietary, genetic, and environmental factors[4]. These stones not only impose a direct clinical burden but also plays a quintessential psychological and financial stress on patients due to frequent hospital visits, diagnostic tests, and invasive treatments [5], [6].

1.1.1 Epidemiological Overview

The occurrence and frequency of kidney stone disease have been on a steady rise globally, primarily due to dietary changes, climate, genetic predisposition, and stagnant lifestyles. According to epidemiological studies, kidney stone generally ranges from 1 - 15% depending on geographic, ethnic, and environmental factors [7] . Developed countries gravitate to report higher prevalence, attributed to high-protein diets, increased consumption of oxalate-rich foods, reduced water intake, and a higher rate of metabolic malfunction such as obesity and diabetes.

In India, the burden of nephrolithiasis is considerable, with estimates indicating that about 12% of the populace suffers from kidney stones, and close to 50% of these individuals may experience reversion within a decade, if mitigative measures are not taken [8]. The disease frequently affects men more than women and is most observed in individuals between the ages of 20 and 50 [9], [10].

1.1.2 Pathophysiological mechanism of kidney stone

Kidney stone genesis, scientifically termed nephrolithiasis or urolithiasis is a type of pathological biomineralization, where mineral deposits abnormally create a stockpile in

the urinary tract or kidneys. Although kidney stones can vary in type and composition, the underlying pathophysiological mechanisms leading to their development generally follow a similar trend: urine supersaturation, nucleation, growth, aggregation, retention, and ultimately leading to stone formation. This sequence generally represents a convoluted interplay of physicochemical, biochemical, and cellular-level adaptations in the urinary environment [1], [11].

1.1.2.1 Supersaturation: The Starting Point of Stone Formation

The supersaturation of urine begins with a condition where the concentration of stone-forming solutes such as uric acid (UA), phosphate, calcium, oxalate and cystine exceeds their solubility limits. In conventional physiology, urine remains undersaturated due to the contiguity of natural inhibitors and sufficient dilution. However, when there is dehydration, increased solute excretion, or reduced urine volume, this balance is disrupted, resulting in supersaturation [12], [13].

Supersaturation is a prerequisite for crystallization and occurs silently over time. Even before stones are clinically visible, subclinical crystalluria (presence of crystals in urine) may occur. The level of supersaturation determines not only the likelihood of stone formation but also the variety of stones that will form. For example, uric acid stones are promoted in acidic urine with high uric acid content, whereas calcium oxalate stones form more readily in concentrated urine with high calcium or oxalate levels [1], [14].

1.1.2.2 Nucleation: Formation of Seed Crystals

When the urine is supersaturated, small particles begin to form through a process called nucleation. This can be:

- Homogeneous nucleation: where solutes spontaneously accumulate together.
- Heterogeneous nucleation: where crystals form on pre-existing substrates like cell debris, urinary casts, or Randall's plaques i.e. calcium phosphate deposits in renal papillae.

Heterogeneous nucleation is utterly common in vivo and is facilitated by factors like oxidative stress, epithelial injury, or urinary tract infections. Nucleation commences from dissolved solutes to solid phase crystals, making it as an essential step in lithogenesis [15].

1.1.2.3 Crystal Growth and Aggregation

After nucleation, seed crystals grow by combining more ions from the supersaturated urine present in the solution. The rate of growth primarily depends on many factors:

- The degree of supersaturation: Higher supersaturation enhances crystal growth by supplying more solutes for deposition[12], [13]
- Presence or absence of inhibitors like citrate, magnesium, and glycosaminoglycans (GAGs) [11], [12]
- pH of urine, which determines the ionization state of solutes Acidic urine stimulates uric acid crystallization, on the other hand, alkaline urine favours calcium phosphate stones [16]

Crystals may also aggregate, forming larger clusters through electrostatic interactions or adhesion to organic matrices like damaged renal cells. Aggregation accelerates stone growth and increases obstruction risks. While crystals smaller than 5 μ are often flushed out by urine flow, those exceeding this minimum limit especially when aggregated overwhelm the kidney's self-clearing mechanisms, leading to retention and clinical stone formation [13], [17]

1.1.2.4 Crystal Retention and Epithelial Adhesion

Crystal retention in the kidneys is an essential step in altering microscopic particles into problematic stones. In healthy individuals, these small crystals are typically washed away by the urine flow. However, retention occurs when:

- Attachment to damaged tissue: Crystals getting sticked to injured epithelial surfaces in the renal tubules.
- Embedding into membranes: Particles accumulating into the kidney's structural layers, such as tubular basement membranes.
- Nesting into Randall's plaques: Calcium phosphate deposits that use to exist before, beneath the kidney's surface behave as "anchors" for crystals [13], [15].

Once crystals get trapped, they can be swamped by kidney cells, leading to oxidative stress and release of inflammatory signals like IL-6 and TNF-α [18], [19] which worsens the

tissue damage, creating a vicious cycle: retained crystals causes inflammation, which further promotes crystal adhesion and growth [20].

1.1.2.5 Role of Promoters and Inhibitors

The balance between crystallization promoters for e.g. calcium, oxalate, uric acid and natural inhibitors for e.g. magnesium, citrate, Tamm-Horsfall protein critically determines the stone formation. Even in supersaturated urine, sufficient inhibitors can restrict crystal nucleation and aggregation. Conversely, less inhibitor levels drastically increase stone formation risk. For instance, citrate binds calcium into soluble complexes, reducing surplus calcium available for stone formation [21]. Magnesium scrambles oxalate crystallization by competing for binding sites [22]. Tamm-Horsfall protein known as uromodulin coats crystals, resulting in clumping and adhesion to kidney tissues [23].

1.1.2.6 Final Stone Formation and Clinical Symptoms

When retained crystals continue to grow unchecked, they eventually become large enough to be considered as renal calculi. These stones may remain asymptomatic and causes:

- Flank pain (renal colic), frequently described as waves of severe, cramping pain.
- Haematuria (blood in urine), visible or detectable only under a microscope.
- Urinary obstruction, restricting urine flow and resulting in pressure accumulation.
- Hydronephrosis results in swelling of the kidney due to urine retention.
- Infections or life-threatening urosepsis results when the bacteria invade the obstructed tract.

At the any of these stages, treatment becomes essential and option ranges from pain management and hydration for small stones to surgical procedures like lithotripsy or ureteroscopy for larger, obstructive stones. The approach depends on the kidney stone's dimension, composition, and location within the urinary system [24], [25].

1.1.3 Classification of Kidney Stones

Kidney stones commonly known as renal calculi, vary tremendously in their morphology, dimension, and mineral composition, which primarily stems from differences in urinary biochemistry. Factors such as solute concentration, urine pH, and the absence or presence

of crystallization promoters or inhibitors determine the type of stone formed[26]. Based on their mineralogical characteristics and pathogenesis, kidney stones are generally classified into five main types: those formed from calcium, uric acid , struvite , cystine, and drug-induced stones [27],[12].

Calcium based stones are the most prevalent type, constituting around 70–80% of all cases of nephrolithiasis[27], [28].These may occur as either calcium phosphate (CaP), calcium oxalate (CaOx), or mixed compositions. Among calcium oxalate stones, the more prevalent subtype, exist in two forms namely, monohydrate (COM) and dihydrate (COD) with COM being more thermodynamic stability and more frequently detected in clinical specimens. Several urinary abnormalities such as hypercalciuria, hyperoxaluria, hypocitraturia, hypomagnesuria, and elevated uric acid levels, along with insufficient hydration, contribute to their formation[28], [29]. CaOx stones typically form in mildly acidic urine (pH 5.0–6.5), whereas CaP stones, often composed of hydroxyapatite or brushite, they are linked to alkaline urinary environments (pH > 7.5) and commonly seen in renal tubular acidosis or chronic urinary infections [30]. These stones are prone to recurrence, necessitating long-term preventive strategies.

Uric acid stones, accounts for 3–10% of cases, which are more common in men and individuals with metabolic syndromes, insulin resistance, diabetes, or gout [31], [32]. These stones form in acidic urine i.e. pH < 5.5, where uric acid, a less soluble end product of purine metabolism undergoes crystallization. Humans are more prone to stone formation because Unlike other mammals, they do not produce uricase, enzyme that transform uric acid into the more soluble compound allontoin [32]. Consuming foods high in purines such as red meat, seafood, dehydration, and low urine output are contributing factors.[33] While uric acid stones do not appear on conventional X-ray images as they are radiolucent, they can be frequently medically managed by alkalinizing the urine using potassium citrate or sodium bicarbonate[34], [35].

Struvite stones are also termed as infection related stones, comprising 10–15% of all kidney stones and they are linked with chronic urinary tract infections triggered by urease-producing bacteria including Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa[27]. Urease catalyses the hydrolysis of urea into carbon dioxide and ammonia, thereby rise in urinary pH and enhancing the precipitation of magnesium ammonium phosphate crystals. These stones can enlarge drastically and develop into staghorn calculi,

occupying major portions of the renal collecting system[36]. They are more commonly observed in women and require a synergistic combination of surgical removal and long-term antimicrobial therapy to shun the recurrence[26].

Cystine stones are scarce, and they found that these account for fewer than 2% of cases. They arise from cystinuria, a genetic disorder inherited in an autosomal recessive manner, resulting from mutations in the *SLC3A1* or *SLC7A9* genes, which weakens cystine reabsorption in the renal tubules [37]. As cystine is less soluble in urine, primarily under acidic conditions, its stockpile leads to crystallization[1]. These stones frequently appear in adolescence and are known for their tendency to repeat. Its treatment involves high fluid consumption, urine alkalinization, and thiol based medications like tiopronin or D-penicillamine that form more soluble disulfide complexes [37]

Drug-induced stones represent around 1% of all the kidney stones cases and result either by the precipitation of sparingly soluble drugs or from drug-induced metabolic disturbances that changes calcium or uric acid concentration. Trivial medications implicated in such stones include indinavir and atazanavir (protease inhibitors used in HIV therapy), triamterene (a potassium-sparing diuretic), and sulphonamide antibiotics [38]. These stones can be radiolucent and are harder to detect without prior advanced imaging, and management often requires cessation of the offending drug along with assistance such as hydration and metabolic correction [39]

While calcium oxalate stones remain the most studied, significant research gaps still exist in understanding of uric acid stone formation mechanism. Most studies have focused on strategies for dissolution and urine alkalization, while the fundamental processes of uric acid nucleation and crystal growth remain poorly unexplored. This underlines the need for further investigation into non-pharmacological approaches, including the usage of natural compounds or phytochemicals, to inhibit early-stage crystallization and reduce recurrence.

1.2 Uric Acid Stone

Uric acid (UA) stones ranks as the second most prevalent form of kidney stone disorder, following calcium-based stones**.** In many animals like birds and reptiles, uric acid serves to eliminate excess nitrogen from the body. However, in most other species, uric acid is produced as an end-product of purine metabolism. These animals possess an enzyme called uricase, which dissociates uric acid into allantoin, a more miscible compound that is then

release in urine. Humans and other higher primates have lost this enzyme over the course of evolution, leading to naturally higher levels of uric acid in the body. Some researchers believe this evolutionary change may have offered certain survival benefits[40]. However, elevated uric acid levels are also connected to health problems such as gout and kidney stones. Despite its clinical relevance, the exact process by which uric acid stones form is still not fully clear. Key contributing factors that have been identified so far includes excessive uric acid which is present in urine i.e. low urine volume, hyperuricosuria, and persistently acidic urinary pH[32]. Uric acid (UA) kidney stones represents the second most frequently occurring type of kidney stone disorder, following calcium-based stones**.** In many animals like birds and reptiles, uric acid serves to eliminate excess nitrogen from the body. However, in most other species, uric acid is produced as final product of purine metabolism. These animals possess an enzyme called *uricase*, which dissociates uric acid into allantoin, a more soluble compound that is then excreted in urine. Humans and other higher primates have lost this enzyme over the course of evolution, leading to naturally higher levels of uric acid in the body. Some researchers believe this evolutionary change may have offered certain survival benefits[40]. However, elevated uric acid levels are also connected to health problems such as gout and kidney stones. Despite its clinical relevance, the exact process by which uric acid stones form is still not fully clear. Key contributing factors that have been identified so far includes excessive uric acid present in the urine i.e. hyperuricosuria, less urine volume, and persistently acidic urinary pH[32].

The metabolism of uric acid (UA) is a multi-step and finely regulated process. In animals, nitrogenous waste is primarily generated through the breakdown of amino acids and purines. When amino acids are metabolized, they release ammonia, a substance that is toxic at elevated levels. To prevent toxicity, the body either converts ammonia into glutamine or processes it into urea for safe elimination. On the other hand, purines, which come from cell turnover, biosynthesis, or dietary intake, are eventually broken down into uric acid, with xanthine oxidase being the final enzyme in this conversion pathway[32].

In most animals, uric acid doesn’t accumulate because it is further broken down into allantoin by an enzyme called uricase. However, in homosapiens and other higher primates, this enzyme has been lost over the course of evolution, resulting in the direct excretion of UA. At the body’s normal pH, uric acid primarily exists in its ionized form, urate, which circulates freely in the blood[41].

On average, the human body produces around 300 to 400 mg of urate daily, although this amount can vary depending on dietary habits. The kidneys are responsible for removing about 70% of this urate, while the remaining portion is eliminated through other pathways, including the gastrointestinal tract. In the gut, certain microorganism breakdown urate into carbon dioxide and ammonia, some of are absorbed back into the body, while the rest are expelled [41].

Although uric acid is easily filtered by the glomeruli in the kidneys, only about 10% of it is actually excreted in urine. This is because most of the filtered urate is absorbed again and secreted again at different stages of the nephron. The S1 segment of the proximal tubule reabsorbs the majority of UA through the URAT1 transporter, which is encoded by the SLC22A12 gene[41]. Later, in the S2 segment, the secretion of urate is facilitated by transporters such as URAT, OAT1, and OAT3. Finally, the S3 segment reabsorbs urate once more, again primarily through URAT1[42].

The prevalence of uric acid (UA) nephrolithiasis is affected by a range of factors, which includes age, sex, geographic region, and environmental conditions. However, there are three primary contributors which are consistently associated with the production of UA stones: persistent low urinary pH, and low urine volume and hyperuricosuria [43]. Among these, urinary pH plays a quintessential role, as UA solubility is highly dependent of pH. When pH of urine drops below 5.5, the solubility of UA decreases exponentially, increasing the likelihood of stone formation [44].

Consequently, urinary alkalization has become the cornerstone of both therapeutic and preventive strategies for UA urolithiasis. Alkali therapy involves raising urine pH to enhance UA solubility and facilitate dissolution of existing calculi. This can be achieved using alkalinizing agents such as sodium bicarbonate or citrate salts. Among these, citrate, particularly potassium citrate, is preferred due to its additional benefits, such as avoiding the generation of gastric CO_2 and the resultant gastrointestinal discomfort that may occur with bicarbonate therapy [44]. Moreover, potassium salts are favoured over sodium or calcium salts to prevent the formation of relatively insoluble sodium urate or calcium urate complexes at elevated pH levels [45]. The recommended daily dose of potassium citrate may be up to 100 mEq, with the target urine pH maintained in the range of 6.0 to 6.5. It is crucial to avoid excessive alkalization, as a urine pH above 6.5 may promote calcium phosphate precipitation [45].

Although urine naturally contains a variety of substances that can inhibit the formation of crystals, the precise role and mechanisms of these inhibitors in uric acid (UA) crystallization and stone development remain poorly understood. Experimental in vitro studies have demonstrated that certain urinary components such as glycoproteins, glycosaminoglycans (GAGs), and surfactants can suppress UA crystal growth. Additionally, bioactive compounds like theobromine and 3,7-dimethylxanthine (DMX) have shown promising inhibitory effects on UA crystallization [46], [47]. Despite these findings, the molecular-level mechanisms by which these inhibitors interfere with nucleation, crystal growth, or aggregation of UA crystals are yet to be fully elucidated.

The UA metabolism is a finely regulated multi-step process. Nitrogenous waste in animals is essentially produced by the deamination of amino acids and purines. When amino acids are catabolized, they break down with the release of ammonia, a compound that is toxic in high concentrations. The body thus converts ammonia to glutamine or metabolizes it to form urea for safe excretion. On the other hand, purines, which come from cell turnover, biosynthesis, or dietary intake, are eventually broken down into uric acid, with xanthine oxidase being the final enzyme in this conversion pathway [32].

In all other animals, uric acid doesn't build up since it's further catabolized into allantoin through an enzyme termed uricase. In humans and other higher primates, though, this enzyme has been lost during evolution to leave the direct excretion of UA. Under the body's usual pH, uric acid is mostly found as its ionized version, urate, which freely circulates in blood [41].

The average human body generates approximately 300 to 400 mg urate/day, which can fluctuate with an individual's dietary consumption. The kidneys excrete approximately 70% of urate, and the rest is expelled through other means, such as the gastrointestinal system. In the intestines, urate is broken down by some bacteria into the carbon dioxide and ammonia, of which some are absorbed again into the body, while the rest are excreted [41].

Though uric acid is filtered free by the glomeruli of the kidney, only 10% of it is excreted in urine. This is due to the fact that the bulk of the filtered urate is absorbed again and secreted at various points along the nephron. The S1 segment of the proximal tubule reabsorbs most of the UA by the URAT1 transporter, which is encoded by the SLC22A12

gene [41]. Subsequently, during the S2 segment, excretion of urate is enabled by the transporters like URAT, OAT1, and OAT3. Lastly, the S3 segment reabsorbs urate again, once again mostly through URAT1 [42].

1.3. Natural Inhibitory Potential of Medicinal Plant for Crystallization

The investigation of medicinal plants as natural inhibitors of crystallization and possible stone-dissolving agents has gained growing scientific attention over the last decade. These plants have been found to possess a vast array of bioactive compounds with a melange of pharmacological activities, which includes anti-inflammatory, antioxidant, and antimicrobial activities [48]. Though mounting evidence of their therapeutic activities, the exact mechanisms by which these phytochemicals affect uric acid (UA) crystals are not yet clearly understood.

Of the medicinal plants examined, *Trachyspermum ammi* (also referred to as Ajwain) has produced favourable litholytic and diuretic properties. Historically, T. ammi has been used in the prevention of calcium oxalate crystal formation and is also said to promote diuresis and inhibit kidney stone recurrence [49], [50], [51]. But although the antiurolithiatic activity of various plants has been investigated against calcium-containing stones, their particular action on inhibiting UA crystallization has been rather less in focus in the scientific literature.

Considering the scarcity of specific studies, with more emphasis on in vitro studies of UA stone inhibition by medicinal plants, further research is called for. The purpose of this work is to make its contribution to the growing evidence in evaluating the medicinal plants' potential, namely T. ammi in prevention of UA crystallization. With further development in analytical methodologies and phytochemical profiling techniques, research in the future may uncover greater understanding of the mechanisms by which plant-derived bioactives influence crystal nucleation, growth, and aggregation. This in turn would lead to the discovery of new, plant-derived preventive or therapeutic measures for uric acid urolithiasis

1.4. Thesis Outline

This thesis is organized into five chapters, each stating a specific aspect of the research focused on kidney stone disease (KSD), especially uric acid (UA) crystallization, and the potential of medicinal plants as natural inhibitors. Chapter 1 introduces the background of kidney stones, discussing their various types of kidney stone, underlying causes, and the importance of UA stone formation. It also showcases the rationale behind exploring medicinal plants for their litholytic and diuretic properties. Chapter 2 provides a comprehensive review of the literature related to UA crystallization and the biological and chemical inhibitors that influence this process. It also synthesizes the prior studies to establish the research gap and narrow down the scientific foundation for the current investigation. Chapter 3 details the experimental methodologies adopted, including the in vitro UA crystallization procedures and the phytochemical characterization of selected medicinal plant extracts. Chapter 4 represents the results and discussion of the study, analysing the effectiveness of multiple plant extracts in inhibiting UA crystallization and interpreting the findings in the context of existing research. Finally, Chapter 5 summarizes the key conclusions drawn from the study and suggests directions for future research, highlighting the scope for developing plant-based therapeutic strategies for the prevention and management of uric acid kidney stones.

2 LITERATURE REVIEW

The formation of kidney stones starts with the process known as crystallization of certain solutes presents in urine. Chemical compounds such as calcium oxalate, calcium phosphate, and uric acid (UA) can undergo crystallization under the favourable physiological conditions. When these solutes exceeds their solubility limit, they begin to precipitate out of the urine, resulting in the formation of microscopic crystals. These crystals might pose a clinical concern if they attach to the renal epithelium, the cellular lining of the kidney tubules or if they accumulate within the narrow structures of the nephron, in the kidney's functional unit. Once embedded, these crystals can act as nucleation sites, promoting the aggregation of additional crystalline material and gradually forming larger masses. Over time, these aggregates can grow in size and develop into clinically significant kidney stones. Consequently, regulating urinary supersaturation and minimizing conditions conducive to crystal formation are critical strategies in the prevention of nephrolithiasis [48]

While a substantial body of research on urolithiasis exists, it dominantly emphasizes calcium oxalate stones, whereas uric acid (UA) stones, despite accounting for a major portion of kidney stone case remain comparatively underexplored. The current literature reveals a scarcity of studies focused on the kinetics and inhibition mechanisms of UA crystallization. Most investigations tend to prioritize the dissolution of existing UA stones rather than understanding the initial stage of crystal formation. Additionally, the antiurolithiatic properties of various medicinal plants have been extensively documented in relation to calcium oxalate stones, there is a conspicuous gap in research regarding their effects on UA crystallization. This present study aims to evaluate, via in vitro experimentation, the influence of selected medicinal plants on UA crystal formation. Specifically, the study will assess their impact on induction time (IT) and crystal size parameters. By doing this, research seek to generate new insights into the role of phytotherapeutic agents in modulating UA crystallization, thereby contributing to the development of potential preventive steps for UA stone formation.

2.1. UA Stones And Crystals

The UA crystallization, which lead to disorders like as hyperuricosuria and gout, present majority challenges in clinical management due to its connection with the kidney stone

formation and joint inflammation[49]. Having understood the stated mechanisms of UA crystallization is important for the treatment strategies. This literature review focuses on analysing the contemporary body of research on UA crystallization, with a focus on both in vitro and in vivo studies.

Grases et al. [50] explored the affects of uric acid (UA) on the crystallization process behaviour of calcium oxalate, particularly under the condition that imitate the human renal environment. Using flow system with synthetic urine, they simulated physiological urinary dynamics to research how UA crystals interact with calcium oxalate crystallization. The researchers used inductively coupled plasma atomic emission spectroscopy (ICP-AES) to examine calcium oxalate formation and used scanning electron microscopy (SEM) to check crystal morphology and composition. Their highlights proved a significant heterogeneous nucleation effect, where the presence of UA crystals actively promoted the production of calcium oxalate monohydrate (COM) crystals. This not only suggested that UA not contributes to its own stone formation but also serves as a nucleation core for COM, thereby facilitating mixed calculi development. The presence of UA at the core of separated COM stones, frequently surrounded by organic matter, reinforces its biomineralization role in initiating and propagating stone growth. This dual role of UA both as a stone-forming agent and as a crystallization promoter for the other salts underscores the difficulties of kidney stone pathogenesis and highlights the importance for inhibition strategies that address multi-component systems, not just isolated compounds.

Pradhane et al. [51] examined about how melamine (Mel) affects the crystallization behaviour of uric acid ? Their study showcased that at less melamine concentrations, the pH remained the dominant factor influencing UA crystallization. However, as Mel concentration approached stoichiometric parity with UA, its influence intensified surpassing that of pH as a key driver of crystallization. Characterization through both PXRD and SEM indicate changes in crystal dimension, suggesting melamine role in inhibiting crystal growth. Furthermore, FTIR analysis also confirmed that the reaction between UA and Mel was primarily physical rather than chemical, indicating the non-covalent or surface-mediated effect. This research provides important insights into how exogenous organic molecules, such as melamine, can act as crystallization promoters under specific concentration limit? When compared to dye inclusions like methylene blue or nucleating substrates like existing UA crystals, melamine's influence is highly

concentration-dependent, highlights the importance of molecular ratios and interaction type responsible for UA crystal formation. Such findings are quintessential for developing effective inhibition strategies in the context of uric acid urolithiasis

Sours et al. [52] presented an insightful study exploring how methylene blue, a cationic dye, which interacts with both anhydrous and dihydrate forms of uric acid (UA) crystals ? Their investigation revealed that the dye becomes integrated into the UA crystal lattice at less concentrations during this crystal growth. The anhydrous UA (UA-MB) exhibited facet-specific inclusion, with the dye molecules preferentially embedding along the [001] and [201] growth planes. In contrast, the dihydrate form (UAD-MB) showed a more diffuse and non-specific incorporation, lacking such crystallographic selectivity. This differential behaviour was further examined through structural analysis, which suggested that π–π stacking interactions which occurs between methylene blue and urate ions, stabilized by interstitial water molecules. Despite the similar molecular environments, only the anhydrous form which demonstrated spatially ordered dye integration, hinting at fundamental differences in crystal growth dynamics or surface chemistry between the two UA polymorphs. Although this precise mechanism behind the selectivity remains unresolved, this study focused on the prospects for external organic molecules to become structurally entrapped in UA crystals; a finding with important implications for understanding how impurities or inhibitors might have modulated the UA crystallization in pathological conditions like uric acid kidney stone formation.

Ezell et al. [52] studied exactly how melamine influences the formation of composite uric acid–calcium phosphate (UA–CaP) crystals under the physiological conditions. Their study demonstrated that melamine significantly promotes co-aggregation, resulting in the formation of larger, more stable crystal aggregates. Differential staining showcased that melamine enhanced the integration of both UA and CaP phases, suggesting a combined interaction facilitated by melamine at the molecular interface. Notably, melamine was also found to attenuate the inhibitory action of hydroxycitrate, a known crystallization inhibitor, resulting in reducing its effectiveness. These findings highlights melamine dual role as both a crystallization enhancer and inhibitor disruptor, particularly in mixed-component systems. When compared to Pradhane et al.'s findings on UA–Melamine interactions, this study extends the understanding by showing melamine's potential to multiply the pathological crystal growth even in the presence of therapeutic agents. This highlights the

importance of including the co-precipitation dynamics and interference with inhibitors in the broader context of urolithiasis prevention and the treatment.

2.2 Modulation of UA Crystallisation by Inhibitory Agents

Grases et al. [53] evaluated the in vitro efficacy of N-acetylcysteine and theobromine in dissolving uric acid (UA) stones using artificially synthesised urine at pH 6.5, a value near to therapeutic alkalinization conditions. The experiment was conducted at 37°C, with artificial urine circulated continuously via a peristaltic pump, and stone dissolution assessed by measuring weight loss every 24 hours. Both compounds individually showed moderate enhancement in UA stone disintegration, but interestingly, their combined use did not yield a synergistic effect. The dissolution performance of the mixture closely matched that of theobromine alone, indicating that theobromine is likely the dominant active agent in this setting. The research suggested that theobromine might act as a promising adjuvant in UA stone chemolysis when used with the alkalizing agents such as bicarbonate or citrate. Earlier discussed inhibitors like hydroxycitrate, which melamine disrupts, theobromine offered a more chemically stable and effective pathway for stone dissolution. This can represent a new oral chemotherapeutic strategy for UA stones, particularly in patients where urine alkalization is already part of the treatment regimen.

Carvalho et al. [54] conducted an in vivo investigation using Dalmatian dogs to understand the role of natural urinary inhibitors namely Tamm-Horsfall protein (THP), nephrocalcin, and glycosaminoglycans (GAGs) in uric acid (UA) crystallization. Urine sample was collected from stone-forming and healthy Dalmatians of same age, and analyse for purine derivatives using HPLC. The study confirmed elevated UA excretion levels in all Dalmatians due to breed-specific purine metabolism. However, there was no significant difference in UA levels between stone-formers and healthy controls. The critical distinction, however, was found in urinary inhibitor levels. Stone-forming dogs exhibited significantly reduced excretion of THP and GAGs, both of which are known for the inhibiting crystal nucleation and aggregation. The absence of other notable purine derivatives further emphasizes the importance of these inhibitory proteins in controlling UA crystallization. This research highlight the protective role of THP and GAGs and suggests that deficiency in natural inhibitors may be a key contributor to stone formation in hyperuricosuric conditions. It also validates Dalmatian dogs as an effective animal

model for studying UA stone pathogenesis, especially in the context of impaired crystallization inhibition rather than overproduction of uric acid alone.

Pradhane et al. [55] investigated the inhibitory potential of 3,7-dimethylxanthine (DMX) on uric acid (UA) crystallization in a controlled by experimental setup using synthetic urine at 37°C. Crystallization was monitored through visual detection of turbidity, an indicator of nucleation onset and the induction time (IT) was recorded. The presence of DMX led to a minute change in crystal morphology, changing from the typical plate-like structures to rod-like structure, with the reduction in crystal size and a significant delay in nucleation. Characterization through PXRD and FTIR confirmed that the DMX do not incorporate into the UA crystal lattice, but rather interferes with crystallization during the liquid phase, likely through surface adsorption or solution-phase interaction. This inhibitory behaviour was later supported by a measurable decrease in nucleation and growth coefficients, indicates a thermodynamically unfavourable environment for crystal development in the presence of DMX. The findings position DMX as a non-incorporating yet effective crystallization inhibitor, offered a mechanistically different mechanism compared to agents like methylene blue which incorporates selectively into the crystal matrix or theobromine which promotes dissolution. DMX's has ability to change nucleation kinetics and crystal habit without becoming part of the lattice highlighting its potential as a solution-phase controller in UA stone prevention strategies.

Grases et al. [47] conducted a vivid investigation into the crystallization which inhibiting potential of various methylxanthines, i.e. caffeine, theobromine, theophylline, and paraxanthine, using induction time (IT) as a kinetic marker for uric acid (UA) crystal formation. The study were performed at 37°C using a mixture of synthetic urine and UA stock solution, with the pH adjusted to 4.5 to 4.65 simulating acidic urinary conditions where UA crystallization is most likely occur. Amongst the tested compounds, theobromine showed the most significantly inhibitory effect, significantly delaying UA crystallization, as observed through turbidimetric monitoring. Beyond its preventive role in primary nucleation, theobromine was also tested for its ability to inhibit regrowth of UA calculi fragments remnants from extracorporeal shock wave lithotripsy (ESWL) procedures. These portions were placed in a closed-loop flow chamber imitating renal conditions, and synthetic urine was circulated continuously. Weight measurements over

time showed a notable suppression in fragment regrowth when theobromine was present. This dual action inhibiting both nucleation and post-lithotripsy regrowth positions theobromine as a promising therapeutic candidate for UA stone management. In contrast to DMX and melamine, which influence crystal morphology or promote growth under specific conditions, theobromine offers broad-spectrum crystallization control and is especially relevant for preventing recurrence in high-risk patients..

2.3. Influence of Medicinal Plants on Kidney Stones

Medicinal plants have served as an effective and easily available alternatives for managing various health conditions including urolithiasis. Growing concerns about the side effects and limitations of conventional pharmacological therapies, combining with the increasing medicinal costs, has renewed interest in the plant-based treatments. This resurgence is reflected in the overall demand for natural, plant-derived remedies [56]. Numerous in vivo and in vitro pharmacological studies have confirmed the antiurolithiatic activity of various medicinal plants, particularly against calcium oxalate (CaOx), the most prevalent component of kidney stones.

However, most of these research, have largely overlook uric acid (UA) kidney stone prevention, despite UA being the second most common component in renal calculi. Currently, there exists a notable research gap regarding the potential role of herbal formulations in inhibiting UA crystal formation and growth. This emphasises the essence for focused research into plant-based interventions targeting UA stones, which could provide safer and more cost-effective therapeutic options, especially in populations at risk of hyperuricosuria related stone formation.

Pandhare et al. [57] investigate the antiurolithiatic potential of *Bryophyllum pinnatum* Lam. in a sodium oxalate (NaOx) induced rat model of urolithiasis. Renal calculi were experimentally induced by administering NaOx, after which rats were treated with oral doses of hydroalcoholic extract of *Bryophyllum pinnatum* for 14 days. The research assess a range of physiological and biochemical markers, including urinary oxalate, creatinine, phosphate, and kidney tissue histopathology, alongside serum parameters and kidney homogenate analysis. The findings demonstrated a dose dependent on inhibition of calcium oxalate crystallization, marked by a significant and effectively reduction in

urinary stone-forming constituents and normalization of altered ion excretion patterns caused by NaOx. Histological improvements and biochemical recovery further confirm the extract's therapeutic properties. These results offer robust pharmacological validation for the traditional use of *Bryophyllum pinnatum* in folk medicine and emphasize its therapeutic potential in managing calcium oxalate-based kidney stones. The study focus on remains on CaOx stones, and similar experimental results for its efficacy against uric acid crystallization remains unexplored highlighting a gap in herbal urolithiasis research.

Kumar et al. [58] investigated the antiurolithiatic efficacy of *Peucedanum grandea*, a herb conventionally used in Unani medicine for urinary ailments. Using a well-established ethylene glycol and ammonium chloride-induced rat model of urolithiasis, the study was conducted in male Sprague Dawley rats. The assessment covered multiple biochemical and physiological parameters, including urinary and serum levels of calcium, phosphorus, creatinine, urea, and quantification of calcium oxalate (CaOx) crystals in urine. Additional validation was provided through kidney homogenate analysis and histopathological examination. The treatment with *Peucedanum grandea* extract resulted in a sudden reduction in the levels of stone-forming ions and urinary CaOx crystals across both standard and test groups. A noticeable spike in urine volume was also seen, which may contribute to natural flushing of crystals. Notably, histological sections they did not show any evidence of CaOx deposition, suggested that the formulation provided effective protection against renal crystal retention. These findings affirm the strong antiurolithiatic potential of *Peucedanum grandea* and support its ethnopharmacological use. However like many other medicinal plant studies, the focus remains limited to CaOx stone models, with no current evidence on its impact on uric acid crystallization.

Bauanani et al. [59] investigates the nephroprotective and antiurolithiatic potential of *Paronychia argentea* extract in a Wistar rat model of calcium oxalate-induced urolithiasis. The study focusses on comparing the butanolic and aqueous extracts of the plant's aerial parts to assess their ability to reduce renal crystal aggregation and prevent stone retention. The experimental results shows that both butanolic extract significantly inhibited renal necrosis and urinary stone retention, as evidenced by reduced calcium oxalate deposits. These extracts also help to mitigate elevated serum creatinine and blood urea levels, indicating strong nephroprotective properties. In contrast, the aqueous extract shows comparatively weaker outcomes in both crystal inhibition and renal protection. The study

also observed lack of hepatoprotective effect as seen by increase in liver weight and serum ALT and PAL levels in the APA-treated groups, resulting towards possible hepatic strain under oxalate load. Paronychia argentea particularly its butanolic extract demonstrated considerable promise in inhibiting CaOx crystal retention, along with conventional medicinal claim. However, the findings also underscored the importance of toxicity profiling and organ specific effect, which are crucial while evaluating any phytotherapeutic agent for urolithiasis.

Ms. Sowmya B A et al. [60] investigates the in vitro antiurolithiatic potential of the ethanolic extract of *Saponaria officinalis*, which aims to offer the natural alternative to conventional synthetic drugs. The study evaluated the extract's efficacy using nucleation and aggregation assays, which imitate at the early and end stages of calcium oxalate (CaOx) crystal formation, respectively. At a concentration of 1000 µg/mL, the extract exhibited a 64.2% ± 0.5 inhibition of nucleation, closely comparable to Cystone, well-established reference drug, which demonstrated 67.7% ± 0.8 inhibition. Likewise, the extract demonstrated a 74.2% ± 0.08 reduction in crystal aggregation which nearly matching Cystone's 76.7% ± 0.09. This result shows that S. officinalis possesses strong inhibitory activity on both the initiation and clustering of CaOx crystals. The research concluded that the ethanolic extract of S. officinalis hold a considerable promise as a plant-based antiurolithiatic agent, supporting its potential for the future pharmaceutical development.

Saha et al. [61] investigates the in vitro antiurolithiatic activity of a hydroalcoholic extract of *Bergenia* ciliata by assessing its ability to inhibit the calcium oxalate monohydrate (COM) crystallization in a synthetic urine system. This experiment was focus on quantifying the nucleation and aggregation rates of COM crystals using spectrophotometric analysis, where reductions in turbidity slope indicated inhibition. This study was compare the extract's performance with Cystone, a widely used polyherbal formulation, at similar concentrations. Results demonstrated that B. ciliata extract showed superior, dose-dependent inhibition of both crystal nucleation and aggregation compared to Cystone. In addition, light microscopy revealed that the extract not only reduced the number and size of COM crystals, but also encouraged the formation of calcium oxalate dihydrate (COD) crystals, which are considered less adherent and less damaging to renal tissue. These results showcases *Bergenia ciliata's* potential as a strong natural inhibitor of

CaOx crystallization, offering preventive and reducing crystal dimensions. However, similar to many pre-existing studies, its impact on uric acid (UA) stone formation wasn't explored, signalling a continuous needs to diversify the scope of phytotherapeutic research beyond calcium-based stones

Kaleeswaran et al. [62] investigate that the antiurolithiatic activity of *Pedalium murex* using its ethyl acetate extract, with a focus on struvite crystal inhibition. In vitro experiments revealed that the extract significantly reduced the size, number, mass, and growth rate of struvite crystals, while also enhancing their dissolution. For in vivo validation, Wistar albino rats were induced with magnesium ammonium phosphate (MgO) based urolithiasis and were treated for the 28 days. Rats received 1% of the ethyl acetate extract showed marked improvements in biochemical markers, along with reduced renal damage and better glomerular integrity, which were comparable to the group treated with Cystone. Histopathological evaluations confirmed reduced tissue injury and crystal deposition in the kidneys. This study is famous for being the first to report the dual-phase in vitro and in vivo efficacy of P. murex ethyl acetate extract against struvite stones, expanding the spectrum of plant-based treatments beyond calcium oxalate and uric acid stones. However, its role in UA crystallization inhibition remains unexplored, underscoring the broader gap in phytotherapeutic coverage for non-struvite lithiasis.

Sasidharan et al. [63] investigates the antiurolithiatic potential of Scoparia dulcis by analysing its capability to dissolve calcium oxalate (CaOx) crystals using titrimetric and turbidity-based in vitro assays, with Cystone serving as the standard reference. The study compared both aqueous and alcoholic extracts of the plant. The result shows that the aqueous extract exhibits dominant dissolution potential as compared to the alcoholic extract and even outperformed Cystone in certain parameters. Both the extracts shows a highly significant reduction in solution turbidity, indicating efficient disintegration of crystal aggregates. These study underscore Scoparia dulcis as a promising herbal candidate for the non-invasive dissolution of urinary calculi, particularly CaOx stones. However, this study do not address its effect on uric acid stone type, a limitation was commonly observed across similar plant-based antiurolithiatic evaluations.

Chinnala et al. [64] examine the inhibitory and therapeutic potential of *Solanum virginianum* against ethylene glycol-induced urolithiasis inside the male Sprague Dawley rats. Rats were given aqueous ethylene glycol for 28 days to induce kidney stone

formation. To assess both preventive and curative effects, the ethanolic extract of S. virginianum was administered orally at 200 mg/kg , 400 mg/kg starting from day one to shun and from day 15 for treatment. The usage of the extract drastically reduces ethylene glycol induced renal damage as seen by histological improvement in kidney tissue and the absence of microcrystalline deposits in treated groups. These finding supports S. virginianum's dual mode efficacy in both preventing and reversing renal calculi, suggesting a strong antiurolithiatic potential. The study was confirmed its protective renal effects; the researchers pointed the need for isolation and characterization of the active phytoconstituents responsible for the observed effects. Like many other herbal interventions, the study focused exclusively on calcium oxalate-based stones, leaving its relevance to uric acid or mixed stone types open for future research.

Garg et al.[65] investigates the inhibitory potential of the ethanolic extract *of Ocimum sanctum* (OS) leaves on calcium oxalate (CaOx) crystal formation. Fresh OS leaves was shade dried, powdered, and subjected to Soxhlet extraction to obtain the ethanol-based phytoconstituent-rich extract. The extract was then tested in vitro for its capacity to minimise CaOx crystallization. The results showed that the crystal inhibition potential of the Ocimum sanctum extract was comparable to that of Cystone, a commonly used polyherbal reference drug. These findings suggest that O. sanctum possesses strong prophylactic potential against calcium-based renal calculi and merits further investigation to isolate active compounds and advance therapeutic development. While promising, the study like many others focused solely on CaOx crystals, and did not assess the extract's effect on uric acid or mixed stones, indicating a continued need for broader spectrum evaluations.

Dharmalingham et al. [66] investigates the antiurolithiatic potential of *Melia azedarach Linn* leaf extracts prepared in both aqueous and ethanolic forms in male albino rats with ethylene glycol-induced calcium oxalate (CaOx) nephrolithiasis. The extracts were administered orally in both preventive and curative treatment protocols. The study shows that both extracts were significantly reduced urinary excretion of calcium, oxalate, and phosphate, key promoters of CaOx stone formation. Additionally, the ethanolic extract restored elevated serum creatinine levels, indicating improvement in renal function. These biochemical findings were substantiated by histopathological analysis, which showed improved renal tissue architecture and reduced crystal deposition. The researchers were

attributes the extract's efficacy to a diversified the mechanism of involving crystal formation inhibition, diuretic activity, antioxidant effects, and protection of renal epithelial cells. While the results strongly support the plant's antiurolithiatic action, the study also emphasized the need for further investigations into its safety profile, diuretic potency, and antioxidant properties to confirm its therapeutic viability in humans.

Anjana Krishnan AS et al. [67] investigates the plant derived therapies are gaining prominence as economical and eco-friendly alternatives for managing uric acid (UA) kidney stones. While UA stones constitute a significant subset of urolithiasis cases, research on their prevention especially through natural compounds remains limited compared to calcium-based stones. *Ocimum tenuiflorum L*. also termed as Tulsi which is widely known as a Ayurvedic herb, has shown promising antiurolithiatic properties. A recent in vitro study evaluated its effect on UA crystallization in synthetic urine. Results revealed a concentration-dependent inhibition of crystal growth and nucleation. Specifically at the pH 5, increasing the Tulsi extract concentration from 27.5 to 110 µg/mL which results in a 19 times increase in induction time and a 3 times reduction in crystal size. This study suggested that the Tulsi effectively delays UA crystallization and reduces crystal aggregation. The study highlights Tulsi's potential as a natural anti-crystallization agent. Moreover, the results are based on synthetic urine models, which lack the difficulties of real human urine comprising ions, proteins, and organic compounds that may influence crystallization differently. Therefore, further research is necessary to validate these findings under physiological conditions. Moreover, understanding the pharmacokinetics, bioavailability, and mechanisms of action of Tulsi's active constituents is essential to assess its clinical applicability. Such investigations could help establish O. tenuiflorum as a viable herbal remedy for UA stone prevention..

Kaur et al. [68] Traditional medicine has long employed plant-based remedies for urinary stone treatment, though scientific validation remains limited. Recent research identified an anticalcifying protein (TAP) isolated from the seeds of *Trachyspermum ammi* (L.) a commonly used medicinal plant in South Asia with promising antilithiatic potential. Initially, TAP was shown to inhibit calcium oxalate (CaOx) crystal growth in vitro through oxalate depletion assays. To explore its in vivo effectiveness, a urolithiatic rat model was developed using 0.4% ethylene glycol and 1.0% ammonium chloride for nine days. A treatment group received 2 mg/kg body weight of TAP in addition to the lithogenic agents.

Results demonstrated that TAP significantly reduced urinary CaOx crystal excretion and crystal retention in renal tissues. Furthermore, TAP preserved renal function and mitigated kidney tissue damage compared to the untreated group. Histological analysis confirmed fewer adhered crystals and less epithelial injury in the TAP-treated group, suggesting both preventative and protective roles. These outcomes validate the potential of *T. ammi*-derived TAP in disrupting CaOx nucleation and crystal adherence mechanisms, while maintaining renal structural integrity. Thus, the protein represents a strong candidate for the development of plant-based therapeutics targeting CaOx nephrolithiasis. This study marks a critical advancement in linking ethnobotanical practices with pharmacological evidence and supports further investigations into the bioavailability, molecular mechanism, and formulation of TAP as a clinically viable antiurolithiatic agent.

Khan et al. [69] The raw extract of *Trachyspermum ammi* seeds (Ta.Cr) has shown promising antiurolithiatic effects through diverse pharmacological actions. In a hyperoxaluric rat model, urolithiasis was incorporated using 0.75% ethylene glycol and 1% ammonium chloride. Ta.Cr, administered at doses of 30 and 100 mg/kg, exhibited significant diuretic activity and reduced calcium oxalate (CaOx) stone formation, confirming its curative potential. In vitro experiments further validated these findings. Ta.Cr resulted in the delayed CaOx nucleation and inhibited crystal aggregation which is depends upon concentration, comparable to potassium citrate. Additionally, the extract demonstrated strong antioxidant activity by scavenging DPPH free radicals, similar to standard antioxidant BHT. Importantly, Ta.Cr reduced cell toxicity and lactate dehydrogenase (LDH) release in oxalate-exposed MDCK cells, indicating renal epithelial cell protection. It also relaxed high K^+ and carbachol-induced contractions in isolated urinary bladder strips of rabbit, suggesting antispasmodic properties that may facilitate stone passage. These multifunctional actions diuresis, crystal inhibition, antioxidant activity, cytoprotection, and smooth muscle relaxation highlight Ta.Cr's therapeutic relevance in managing urolithiasis. The findings rationalize its traditional use and point toward its potential as a plant-based, non-invasive alternative for kidney stone prevention.

As with most herbal studies, the extract's effects were explored solely on CaOx stones, and its impact on uric acid or mixed-type urolithiasis remains unexamined.

Uric acid (UA) remains a significant contributor to kidney stone formation, yet its crystallization kinetics and mechanisms have been relatively understudied over the past

two decades. Most existing literature has focused either on the pathophysiological aspects of UA stone formation or on their dissolution, typically through in vivo experiments or survey-based clinical studies. While such in vivo models, using animals or cultured cells, offer valuable insights, they are often associated with ethical limitations. Moreover, research directly exploring the fundamental crystallization process of UA, especially its kinetics, remains scarce, despite its critical role in developing targeted prevention and treatment strategies.

Notably, the research group led by Grases has played a pivotal role in advancing our understanding of UA crystallization inhibitors. There in vitro studies, largely reliant on turbidimetric analysis have contributed significantly to the field. However, turbidimetry, although effective in assessing inhibitory activity, provides limited insights into crystal morphology, size distribution, and growth dynamics, all of which are vital for comprehensively characterizing the crystallization process.

A considerable number of studies have explored the antiurolithiatic potential of medicinal plants, but the majority have been restricted to calcium oxalate (CaOx) stone models. Research specifically focusing on UA crystal inhibition by plant-based compounds is extremely limited. Most of these studies also rely on turbidity measurements, often overlooking detailed crystal characterization and the influence of phytochemicals on nucleation and growth stages.

This research focuses to fill this knowledge gap by scrutinizing the antiurolithiatic efficacy of medicinal plant extracts on UA crystallization through well-structured in vitro experiments. By evaluating parameters such as induction time (IT), crystal size, and morphology, this study will provide deeper insight into how bioactive compounds influence UA crystal formation. The outcomes could contribute to the development of natural, plant-based therapeutic alternatives for managing UA kidney stones, offering a sustainable and less invasive approach in contrast to conventional pharmacological treatments.

3. MATERIALS AND METHODS

3.1. Chemicals Required

All reagents used in this study were employed as received, without any additional purification. Uric acid (UA) was obtained from Sigma-Aldrich Pvt. Ltd. Chemicals such as potassium chloride, ammonium chloride, sodium dihydrogen phosphate, sodium sulphate, acetone, magnesium sulphate heptahydrate, ammonium sulphate, iron oxide, sodium acetate, sodium carbonate, and sulfuric acid were sourced from Merck Life Science Pvt. Ltd. Sodium chloride and chloroform were procured from Thermo Fisher Scientific Pvt. Ltd. Hydrochloric acid was acquired from Finar Limited, and ethanol was purchased from Anil Cottage Pvt. Ltd. Iodine was obtained from Qualigens Fine Chemicals. Quercetin, ascorbic acid, gallic acid, sodium hydroxide, potassium iodide, ammonium molybdate, FCP reagent, and ferric chloride were procured from Sisco Research Laboratory Pvt. Ltd., while aluminium chloride was sourced from Loba Chemie Pvt. Ltd

3.2. Preparation Of Extract

The *Trachyspermum ammi* also known as Ajwain was purchased from the local shop in Nagpur Maharashtra. The seeds were first dried at 40^0 C before being grind into final powder. The dried seeds were grounded using a mixer. The aqueous extract of T.ammi was prepared using the decoction method. Specifically, 25 gram of dried powder was added to 100 ml of distilled water and poured into a neck round bottom flask into a heating mantle. The mixture was heated at 100^0 C for 30 minutes to minimise solvent evaporation, the flask were covered during the extraction process. The extract was collected and cooled down, then the following extract were centrifuge at 10000 RPM for 10 min at 22^0 C. The solvent were collected and called as final extract and which is now ready to use and stored in refrigerator at 4^0 C.

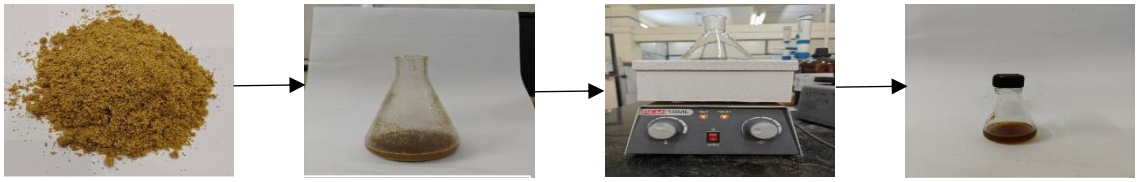

Figure 1: Visual representation of decoction process

3.3. UA Crystallisation

The crystallization of uric acid was performed by the method provided by pradhane et al**.** [51]. For each experiment A fresh stock solution of uric acid was prepared by d dissolving 0.2 gm of UA in a mixture of 95 ml Water and 5 ml of 1M NaOH. Composition of synthetic urine was also provided by pradhane et al. [51]**.** Crystallization experiments were conducted in a jacketed flask, containing 160 ml of synthetic urine each mix with 40 ml of uric acid solution. resulted in a final pH of 7.2 Measured by incorporating Labman LMMP 30 pH meter. A REMI 1 MLH magnetic stirrer was used to ensure homogeneous mixing while temperature was maintained at 37^0 C. The pH was continuously monitored an adjusted using pH electrode hands supersaturation of uric acid was achieved by adding 2 to 3 ml of concentrated HCL. The induction time (IT) for crystallization indicated by the appearance of turbidity was recorded once pH adjustment commenced. Crystallization was also examined in the presence of extract at varying concentration. Resulting crystals were collected into micro centrifuge tubes and preserve for further analysis.

Table 1: Composition of synthetic urine

Compound	Concentration(g/L)
KCl	4.75
NaCl	6.16
$MgSO_4.7H_20$	0.95
NaH_2PO_4	4.45
Na_2SO_4	2.41
NH_4Cl	2.43

Figure 2: Crystallization setup for UA

3.4. Phytochemical Analysis

Phytochemical Analysis Help in the identification of bioactive compound found in plants And to gain insight into their chemical composition. This naturally occurring substance that is bioactive compounds Also known as phytochemicals. These phytochemicals play vital roles in plant growth logical process and defence mechanism of the plant [70]. Analysis was done using following standard Procedure then by [71], [72].

Phytochemical screening procedure

A series of simple chemical test for carried out to check the presence of major phytochemical group present in plant extract.

- Phenols: 2 drops of containing 5% ferric chloride solution then added to 1 ml of aqueous extract. Blue green and black colouration indicated the presence of phenolic compound.
- Alkaloids: To 2 ml of the aqueous extract, 1 ml of diluted hydrochloric acid and then addition of 3 ml of Wagner's reagent took place. The presences of alkaloids was confirmed with reddish-brown precipitate.
- Flavonoids: Mixing 2 ml of the aqueous extract with 3 ml of lead acetate solution , indicates the presence of flavonoids was confirmed by appearance of a yellow precipitate
- Coumarins: Addition of 1 ml of 10% sodium hydroxide into 2 ml of the aqueous extract, A yellow colour appearance indicating the presence of coumarins.
- Saponins: Vigorous shaking of 2 ml of aqueous extract with 3 ml of distilled water for about 10 minutes took placed resulting in formation of froth or foam indicating the presence of saponins.
- Steroids: Addition of 1 ml of the aqueous extract with 2 ml of chloroform, later addition of 2 ml of concentrated sulfuric acid and 1 ml of acetic acid done. A green coloration indicated the presence of steroidal compounds.
- Glycosides: In this test, 1 ml of the aqueous extract was combined with 2.5 ml of glacial acetic acid and three drops of 2% ferric chloride solution. This mixture was then incorporated with 2 ml of concentrated sulfuric acid. the presence of glycosides confirmed by the appearance of a brown ring at the interface.

3.5. Total Phenolic and Flavonoid Analysis

The total phenolic content (TPC) in the aqueous extract, a slightly modification in Folin–Ciocalteu (FCP) method was followed [73]. The FCP reagent, which contains a mixture of phosphotungstic acids and phosphomolybdic acid, which is then reacts with phenolic compounds in an alkaline medium to make a blue coloured complex. This colour intensity, which is directly proportional to the phenolic concentration, was measured using a spectrophotometer at 765 nm [74].

In this procedure, 200 µL of the aqueous extract of T.ammi was first mixed with 5 ml of 10% (w/v) FCP reagent. After a period of five minutes, 4 ml of 7% sodium carbonate

solution and 2 ml of distilled water were added to the mixture. The entire solution was then incubated in the dark at 40°C for one hour. 1 mg/ml of Gallic acid was used as the reference standard, and calibration was done using gallic acid solutions ranging from 50 to 500 µg/ml. The results were measured in terms of gallic acid equivalents (GAE) per gram of dry sample (mg/g), and a standard formula was applied to compute the TPC.

$$C = z \times \left(\frac{1}{\rho}\right) \quad (1)$$

Where in,

C = Total phenolic content g GAE/g dry extract,

z = concentration obtained from calibration curve

ρ = Density of extract

To measure the total flavonoid content (TFC), the aluminium chloride colorimetric assay was employed, depends on the formation of a flavonoid aluminium complex, which induces a measurable colour shift [75]. Quercetin is used as the reference compound, and a standard curve was obtained using concentrations between 100 and 500 µg/ml.

In this method, 500 µL of the Aqueous extract was mixed with 400 µL of ethanol. After five minutes, 500 µL of 2% aluminium chloride solution in ethanol was added. All the reagents are mixed well and incubate at room temperature for 40 minutes. The absorbance was then measured at 510 nm. The TFC was expressed in terms of mg of quercetin equivalents per gram of dry extract (mg/g).

3.6. Total Antioxidant Analysis

The antioxidant Capacity of the aqueous extract was assessed using with some modification of the phosphomolybdate assay, described by [76]. This method based on the principle that antioxidant compounds in the sample can convert molybdenum in its +6 oxidation state (Mo^{6+}) into a reduced form (Mo^{5+}), which results in the appearance of a green-coloured complex.

To prepare the reagent, 0.168 g of sodium dihydrogen phosphate (NaH_2PO_4), 1.6 ml of sulfuric acid (H_2SO_4), and 0.247 g of ammonium molybdate were each dissolved in water and brought up to 50 ml. These solutions were then mixed to make a total of 150 ml of phosphomolybdenum reagent.

For analysis, 3 ml of this reagent were added to 600 µL of the aqueous extract. The mixture was then incubated in a water bath at 90°C for 90 minutes. then allowed it to cool at room temperature, then the absorbance was measured at 690 nm using a spectrophotometer.

Ascorbic acid was used as the reference standard. A calibration curve was created using the mean absorbance values that were found at different concentrations of ascorbic acid that is from 100 to 1000 µg/ml. The antioxidant capacity of the aqueous extract was represented as mg of ascorbic acid equivalent (AAE) per gram of dried sample (mg AAE/g).

3.7. Characterization

The uric acid (UA) crystals collected from the crystallizer were first observed under a light microscope (Optscopes) using a 40X objective lens. A digital camera attached to the microscope captured the crystal images. The size of these crystals was later measured using ImageJ software, and the reduction in size was shown.

To understand the structure of the crystals, powder X-ray diffraction (PXRD) test was done using a Rigaku Miniflex 600 instrument with copper radiation. For identifying the various chemical bonds present in the crystals, an infrared (IR) analysis was carried out using an IRSpirit spectrophotometer (Shimadzu) in the range of 400 to 4000 cm^{-1}.

The morphology of the crystals was examined with a Scanning Electron Microscope (SEM) model JEOL JSM-6380A. The UA sample was fixed onto a sample holder using carbon tape, coated with a thin layer of platinum through a sputtering process, and SEM images were taken at a voltage of 5kV

4. RESULTS & DISCUSSIONS

4.1. Antiurolithiatic Property of *Trachyspermum Ammi*

Trachyspermum ammi, more commonly called as Ajwain or bishop's weed, has a rich history in traditional medicine systems such as Ayurveda and Unani. Traditionally, it has been used to treat issues related to digestion, the respiratory tract, and inflammation, thanks to its rich mix of natural compounds like thymol, flavonoids, and essential oils.While the seeds of T. ammi are celebrated for their broad-spectrum medicinal properties, their role in urolithiasis, its ability to prevent kidney stones has not been studied enough. Some early investigations suggest that extracts from T. ammi may interfere with the development and clustering of calcium oxalate crystals, which are commonly involved in stone formation [77]. and no literature has yet explored its potential inhibitory effect on UA crystallization. This study focuses on the inhibitory potential of T.ammi on UA crystallization.

4.2. Phytochemical Analysis

The phytochemical analysis of the aqueous extract of T.ammi was carried out, and the results obtained are shown in Table 2. The antioxidant property of T.ammi is attributed to the presence of phenols and flavonoids, which have the capability to donate electrons, but the exact mechanism through which phytochemicals present in T.ammi contribute to the antiurolithiatic effect remains unknown and is a subject of future research. The result showed the presence of various secondary metabolites such as phenols, flavonoids, glycosides, saponins, and steroids and alkaloids. The active constituents in T.ammi, particularly thymol is considered the principal bioactive compound responsible for the plant’s antimicrobial, anti-inflammatory, and antioxidant properties, while flavonoids and saponins play key roles in its antiurolithiatic, hypouricemic, and nephroprotective activities [78]. It is also reported that the leaves and seeds of *Trachyspermum ammi* possess notable hypouricemic and uricosuric properties. These effects are largely attributed to its rich phytochemical content, particularly thymol and flavonoids, which may act by inhibiting xanthine oxidase activity and promoting the renal excretion of uric acid.[79]as the potential of T.ammi to reduce the UA formation in the body.

Table 2: Phytochemical analysis of T.ammi extract

Test Tube Label	Phytochemicals	Test	T.ammi extract
a	Phenols	Ferric chloride test	+
b	Alkaloids	Wagner's test	+
c	Flavonoids	Alcoholic ferric chloride test	+
d	Glycosides	Keller Kiliani test	+
e	Saponins	Foam test	+
f	Steroids	Salkowski test	+

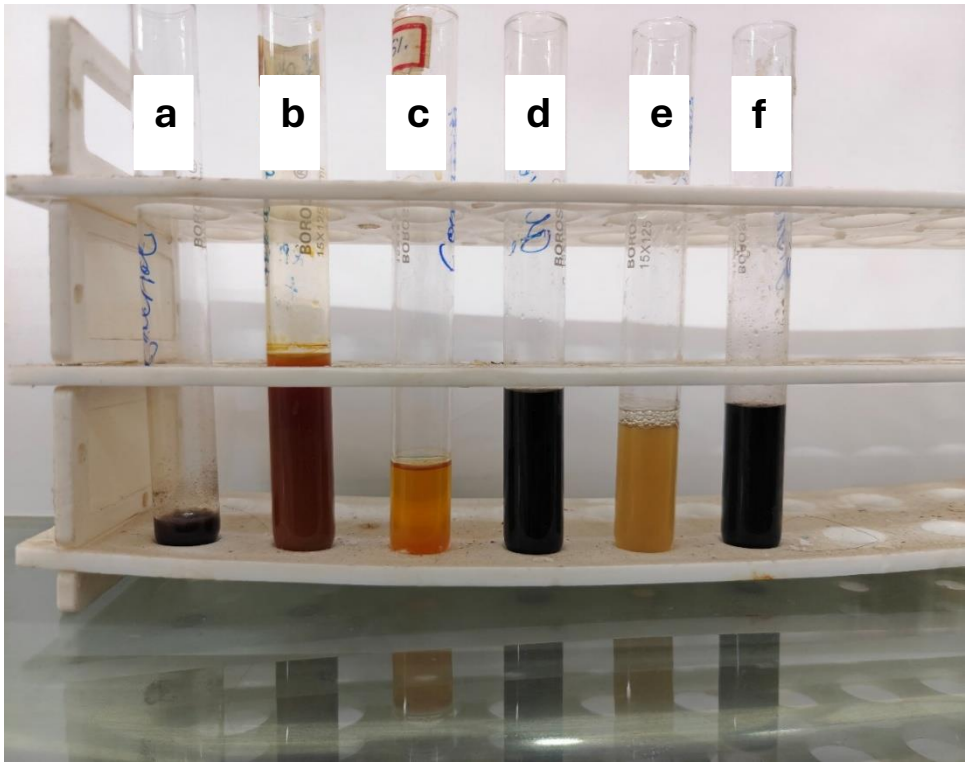

Figure 3: Visual representation of phytochemical analysis

4.3. Total Phenolic and Flavonoid Analysis

The phenolic and flavonoid contents are significant factors in both the quantitative and biological evaluation of the extract [80]. FCP reagent is a mixture of phosphotungstic acids and phosphomolybdic. When the phenolic content in extract reacts with this mixture under alkaline condition it will result in the formation of blue coloured complex which can be quantified by spectrophotometry [74]. The coefficient of determination (R^2) for the calibration curve obtained ($y = 0.0028x + 0.0866$) was 0.9989 as shown in figure 5, indicating good linearity in the investigated concentration range. The total phenolic content in the aqueous extract of T.ammi was found to be 8.89 mg GAE/g. The principle of Aluminium chloride method for flavonoid content determination is in the creation of a complex between aluminium chloride and flavonoids, specifically flavones and flavonoids resulting in colour change. The coefficient of determination (R^2) for the calibration curve obtained ($y = 0.0004x + 0.1205$) was 0.9938 as in Figure 7, and the total flavonoid content in the aqueous extract of T.ammi was found to be 1.206 mg quercetin per g of extract.

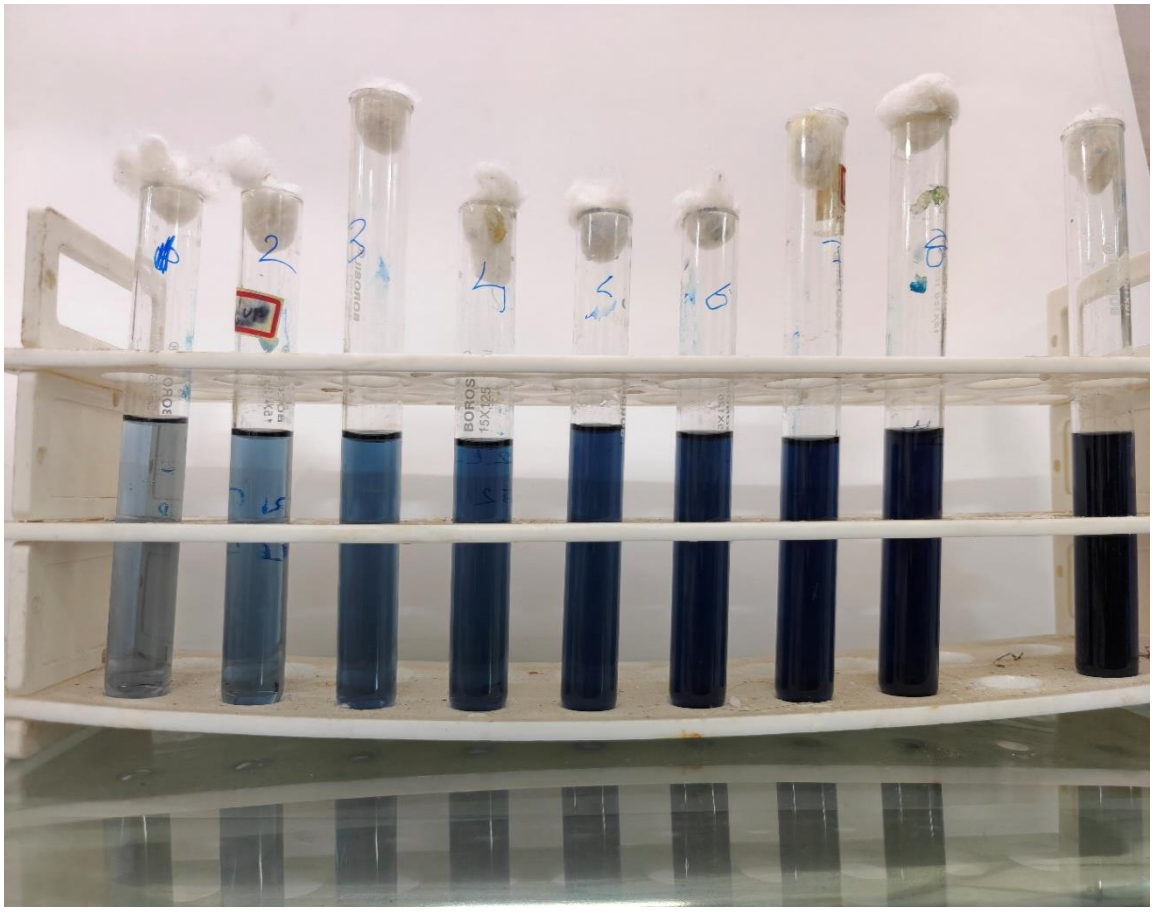

Figure 4: Visual representation of TPC analysis samples

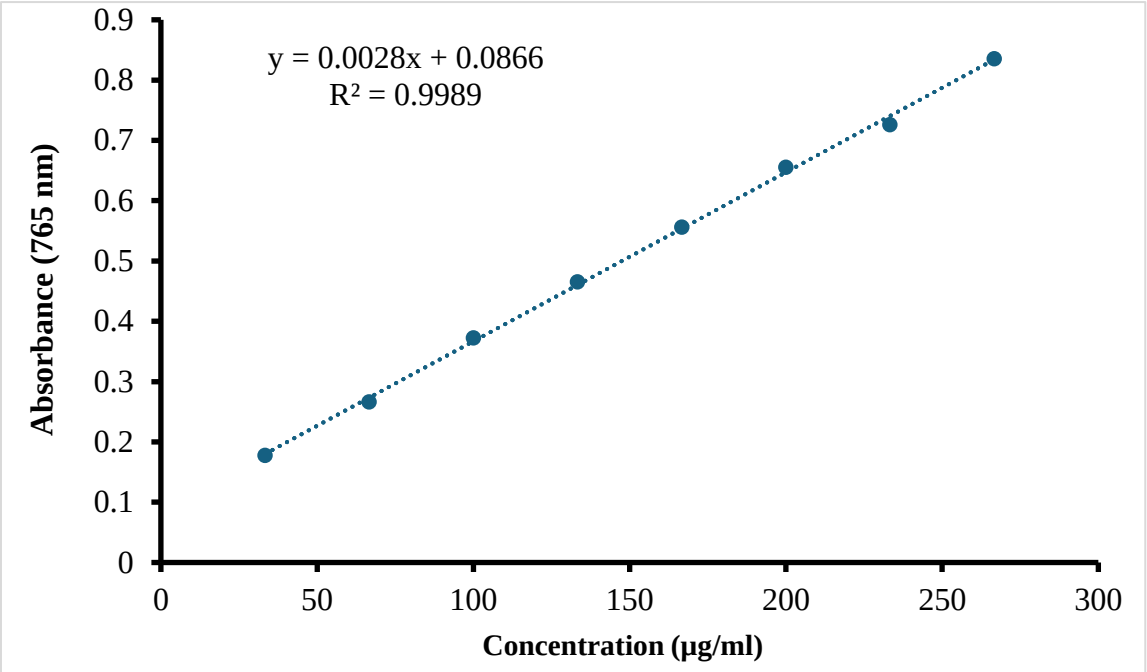

Figure 5: Standard curve for TPC

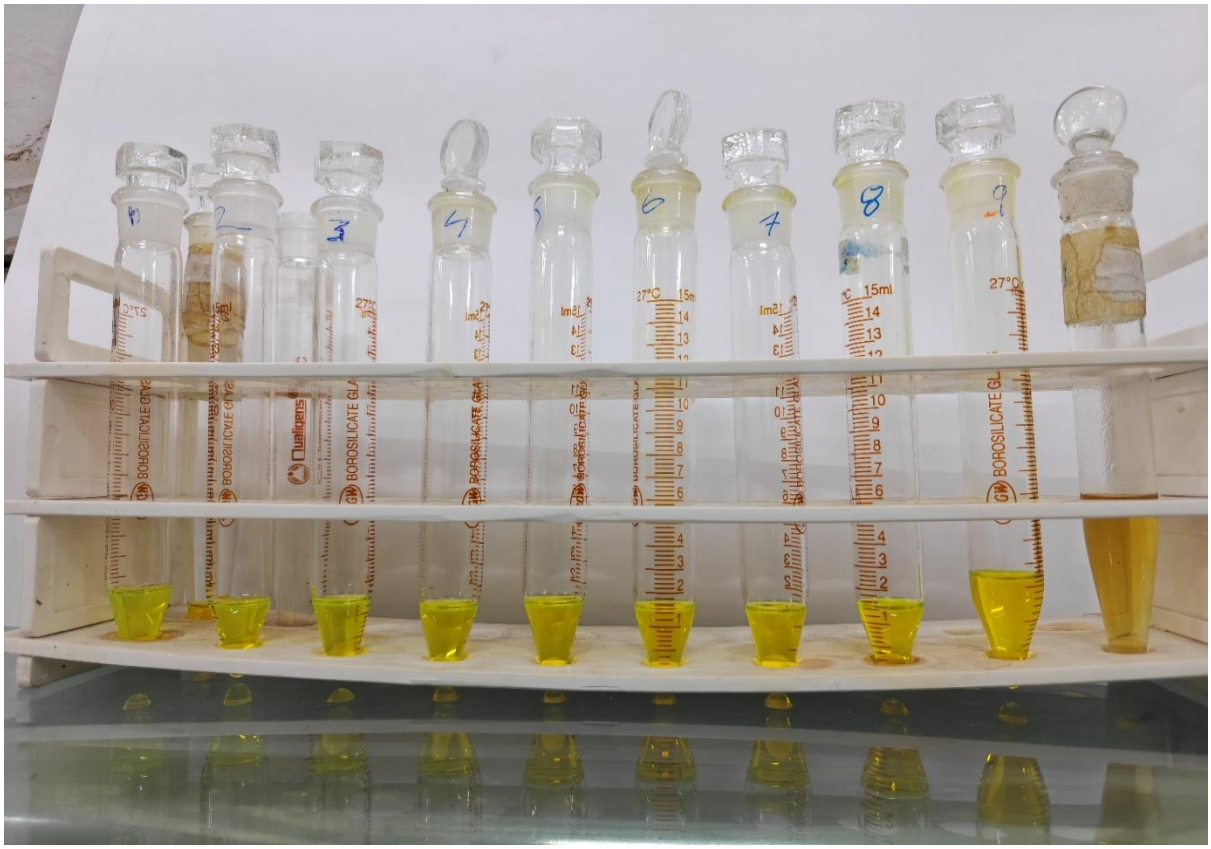

Figure 6: Visual representation of TFC analysis samples

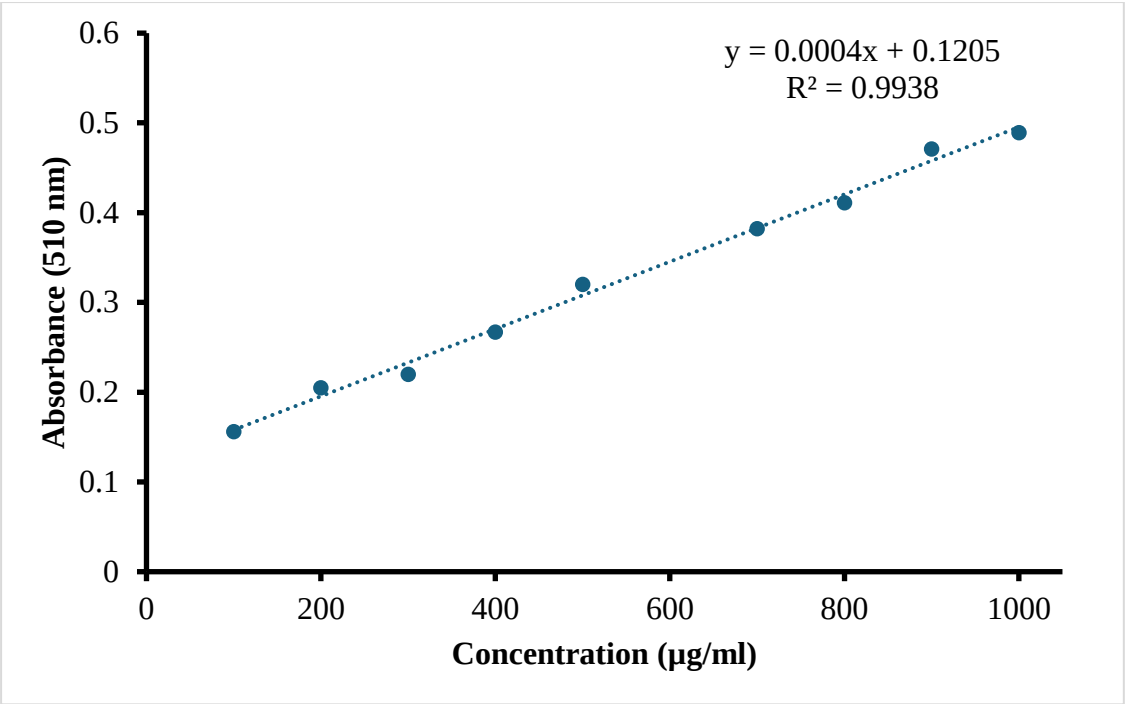

Figure 7: Standard curve for TFC

The result of the aqueous extract of T.ammi showed significant levels of phenolic compounds and flavonoids. Phenolic compounds and flavonoids serve as antioxidants by scavenging free radicals and mitigating oxidative stress in the body. Oxidative stress (OS), characterized by an instability between the generation of oxidants and the defence mechanism of body's antioxidant, has been linked to the development of kidney stones [81]. Numerous studies have underscored the increased levels of oxidative stress observed in individuals with renal stones [82]. It has also reported mitochondrial disfunction in monocytes obtained from kidney stone patients, resulting in increased inflammation and oxidative stress levels [83]. The significant total phenolic and flavonoid content in T.ammi aqueous extract may help in inhibiting UA crystallization and decreasing the chance of kidney stone formation through antioxidant and anti-inflammatory actions.

4.4. Total Antioxidant Analysis

The total antioxidant capacity by phophomolybdate method is based on the principle that the antioxidants present in the extract will reduce the molybdenum (VI) to molybdenum (V) which gives a green coloured complex. The standard curve for the TOC is shown in Figure 9. The coefficient of determination (R^2) for the calibration curve obtained ($y = 0.0031x + 0.0802$) was 0.9928. The total antioxidant capacity was found to be 0.824

mg AAE/g. The results showed that T.ammi extract exhibits significant antioxidant activity, due to the presence of phenols and flavonoids. These compounds effectively neutralize free radicals, indicating that T.ammi can reduce oxidative stress and protect against cellular damage.

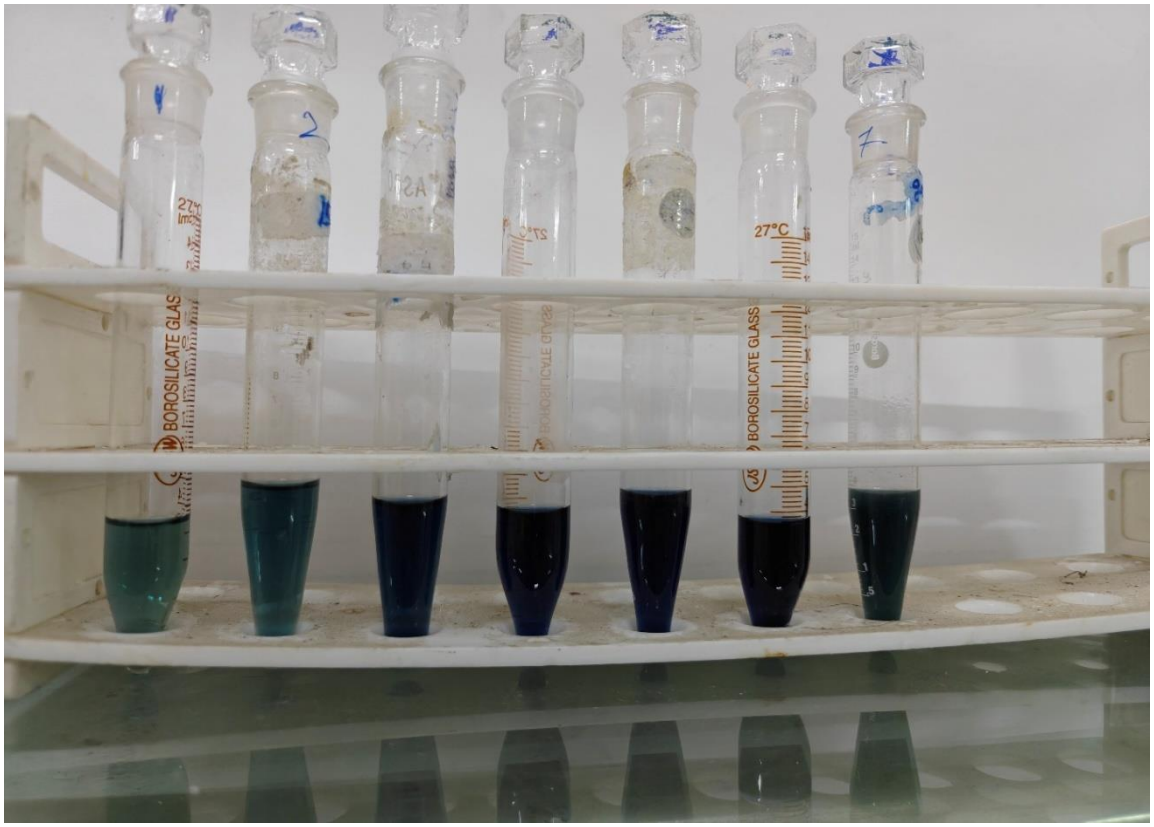

Figure 8: visual representation of TAC analysis samples

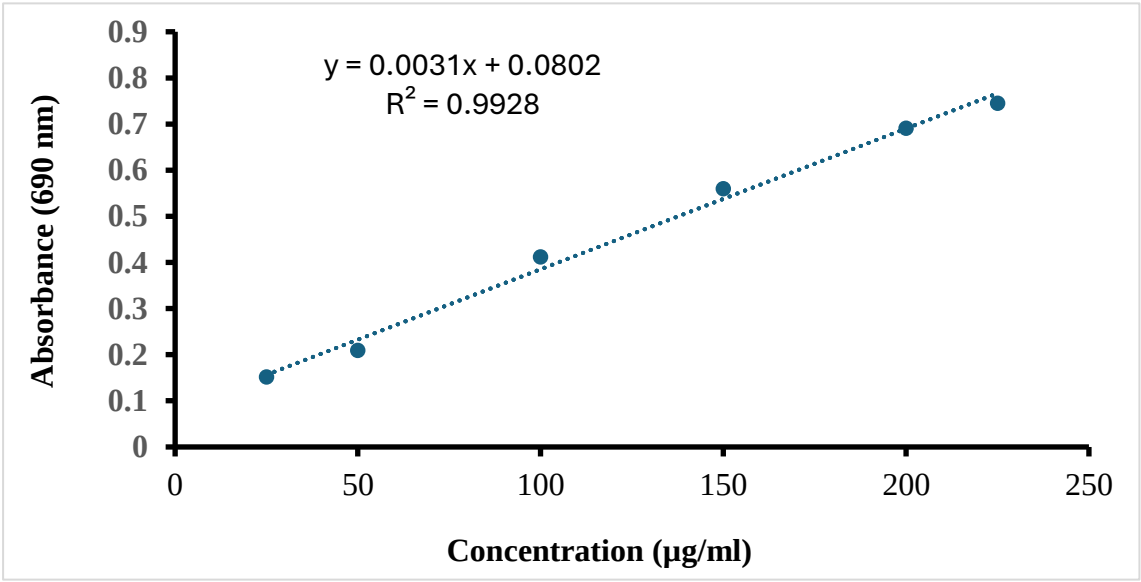

Figure 9: Standard curve for TOC

4.5. Effect of pH and Extract Concentration on Induction Time

The variation in induction time (IT) with increasing concentrations T.ammi extract under different pH conditions i.e. 4.0, 4.5, and 5.0 is presented in Figure 10. Induction time serves as a measure of the time required for the onset of uric acid crystal nucleation, with longer IT indicates stronger inhibition of crystallization.

The data clearly indicate that induction time increases progressively with both pH and extract concentration. At pH 4, only a modest increase in IT is observed. At pH 4.5, a moderate but consistent rise is noted. At pH 5.0, induction time increases dramatically rising from under 510 seconds at 0.32 mg/ml to over 8250 seconds at 2.60 mg/ml confirming a synergistic effect between higher pH and extract dosage

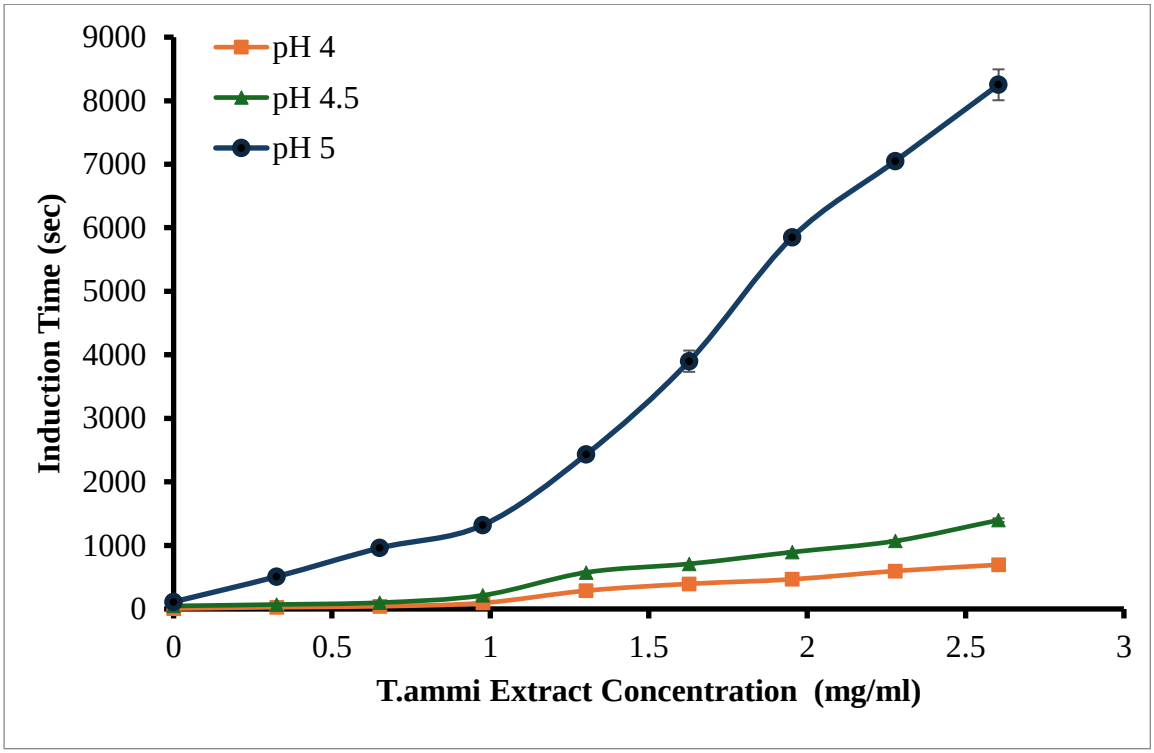

Figure 10: Variation of induction time with extract concentration at different pH levels.

Table 3: Variation of induction time with extract concentration at different pH levels.

Concentration of Extract (mg/ml)	Induction time (sec)		
	pH 4	pH 4.5	pH 5
0	6.5 ± 1.32	47.5 ± 2.17	112 ± 8
0.32	27.5 ± 1.32	70 ± 6.55	510 ± 24.75
0.65	42.5 ± 2.29	100 ± 4	960 ± 8.88
0.97	95 ± 4.58	215 ± 6	1320 ± 51.97
1.30	287.5 ± 4.92	575 ± 14.73	2430 ± 32.78
1.62	395 ± 5.56	710 ± 11.26	3900 ± 167.53
1.95	467.5 ± 10.25	895 ± 14.73	5850 ± 48.49
2.27	597.5 ± 5.76	1070 ± 16.09	7050 ± 50.40
2.60	695 ± 13.74	1397.5 ± 28.83	8250 ± 243.31

4.6. Effect of pH and Extract Concentration on Uric Acid Crystal Size

The influence of T. ammi extract concentration on the mean crystal size of uric acid at varying pH conditions is illustrated in Figure 11. Crystal size is a critical indicator of nucleation and growth dynamics. Smaller sizes reflect enhanced inhibition of aggregation.

Across all pH levels, crystal size decreased consistently with extract concentration. At low concentrations (0–0.32 mg/ml), size dropped sharply from ~0.025 mm to below 0.01 mm. The trend continued at higher doses. Among all conditions, pH 5.0 combined with 2.60 mg/ml extract showed the smallest size (~0.003 mm), highlighting a synergistic inhibitory effect.

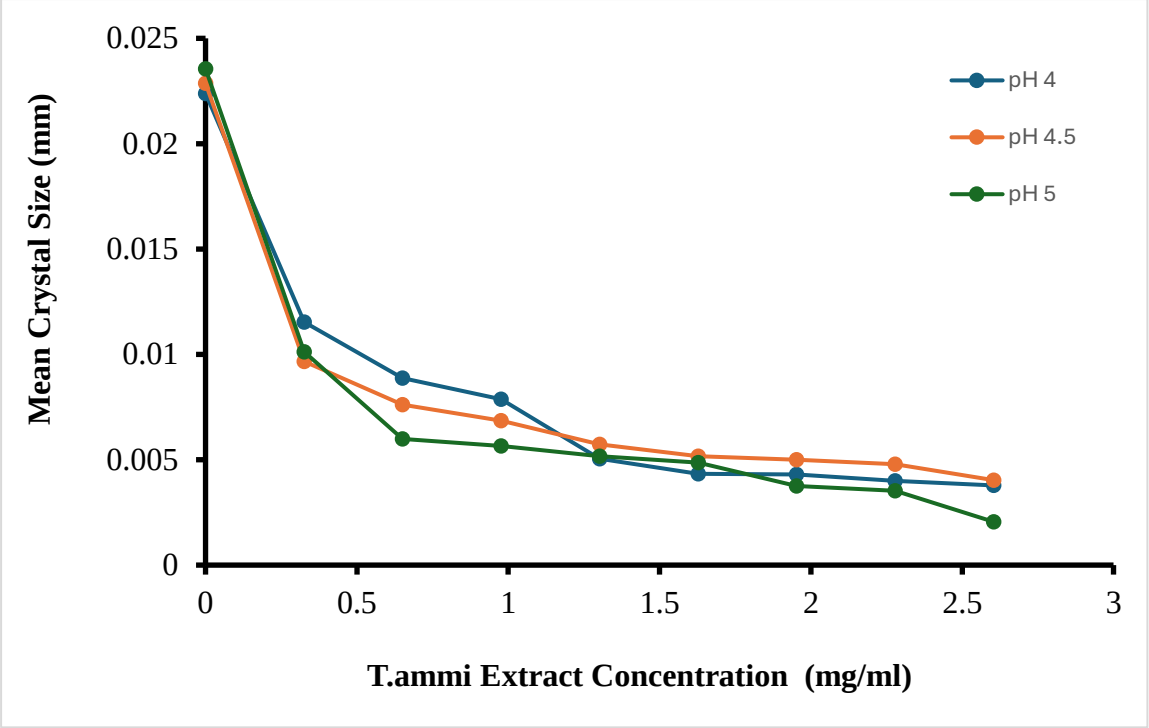

Figure 11: Reduction in uric acid crystal size with increasing extract concentration at different pH levels.

Table 4: Variation of mean crystal size with extract concentration at different pH levels

Concentration of T.ammi extract (mg/ml)	Mean Crystal Size (mm)		
	pH 4	pH 4.5	pH 5
0	0.022385	0.02287	0.023555
0.3255	0.011539	0.009663	0.010114
0.651	0.008867	0.007609	0.005988
0.9765	0.007873	0.00686	0.005648
1.302	0.005051	0.005732	0.005171
1.6275	0.004328	0.00517	0.004864
1.953	0.004297	0.004994	0.003756
2.2785	0.003992	0.004791	0.003523
2.604	0.003782	0.004033	0.002053

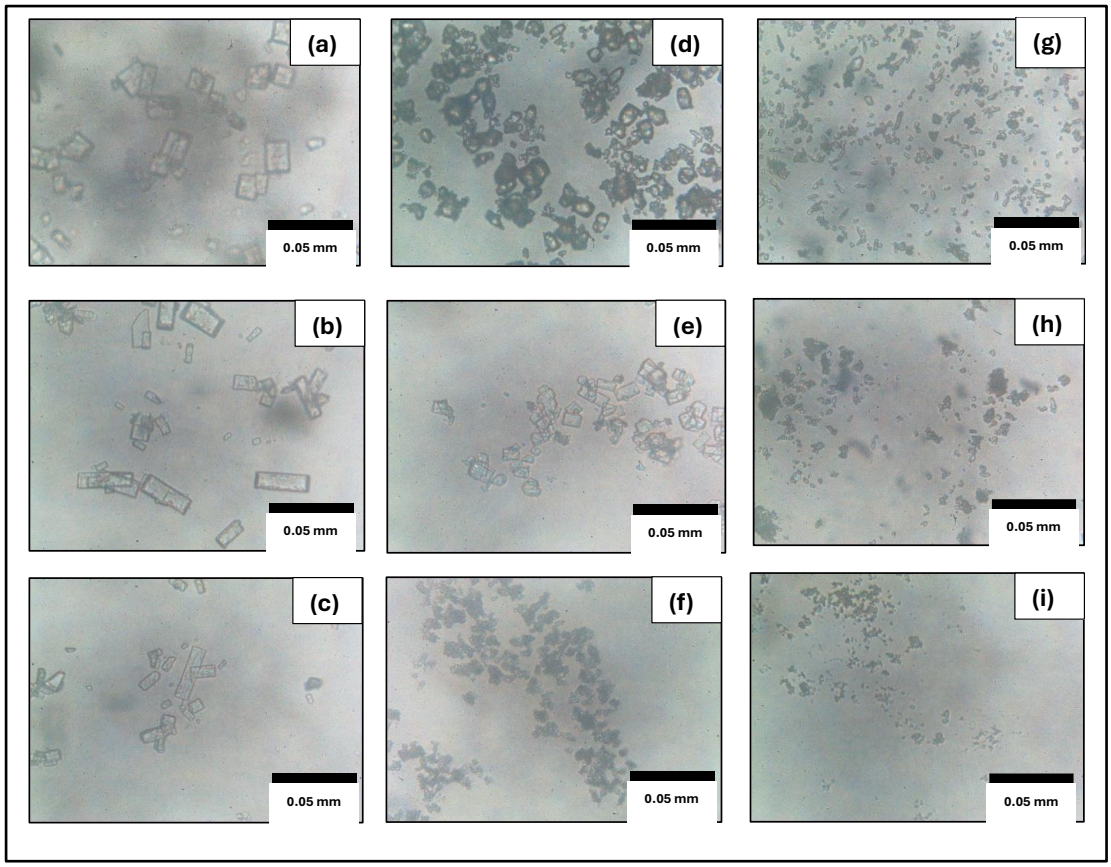

Figure 12: Microscopic images of UA crystals without of T.ammi extract (a) pH 4 (b) pH 4.5 and (c) pH 5; with extract concentration 1.30 mg/ml (d) pH 4 (e) pH 4.5 and (f) pH 5; and with extract concentration of 2.27 mg/ml (g) pH 4 (h) pH 4.5 (i) pH 5.

4.7. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FTIR analysis of uric acid crystals at pH 4, 4.5, 5, both in the absence and presence of T. ammi extract at varying concentrations, reveals that while the principal vibrational bands remain largely unchanged in terms of position, their relative intensities vary across the spectra. The characteristic peaks corresponding to N–H and O–H stretching (~3400–3200 cm^{-1}), C=O stretching (~1700 cm^{-1}), and C–N/C=C stretching in the fingerprint region (~1540 cm^{-1}) are consistently observed in all samples, indicating that the fundamental molecular structure of uric acid remains intact even in the presence of the extract[84],[85][86].

However, minute variations in the band intensity, particularly in the N–H/O–H and C=O regions, suggest changes in the molecular environment likely due to weak intermolecular

interactions such as hydrogen bonding or surface-level adsorption of extract phytochemicals like thymol and polyphenols. These interactions may not significantly alter the uric acid molecule itself but could influence crystal surface characteristics. The increased or decreased absorbance at certain bands especially at the higher extract concentrations, may be indicative of changes in crystallinity or the extent of molecular ordering. Overall, the FTIR spectra implies that T. ammi extract does not chemically modify uric acid but exert its inhibitory effect on crystal formation through mild, non-covalent interactions.

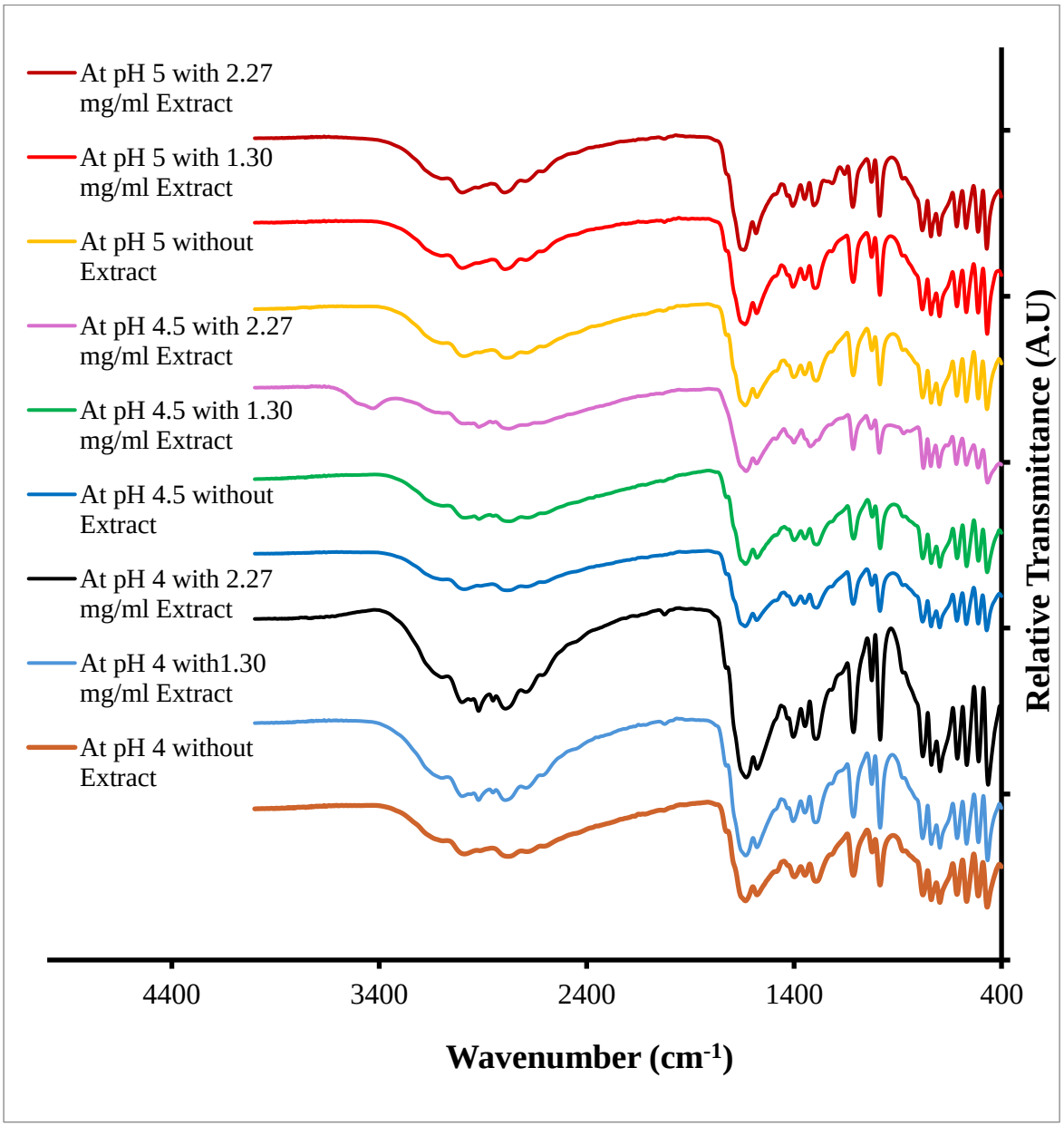
At pH 5 with 2.27 mg/ml Extract
At pH 5 with 1.30 mg/ml Extract
At pH 5 without Extract
At pH 4.5 with 2.27 mg/ml Extract
At pH 4.5 with 1.30 mg/ml Extract
At pH 4.5 without Extract
At pH 4 with 2.27 mg/ml Extract
At pH 4 with1.30 mg/ml Extract
At pH 4 without Extract
Relative Transmittance (A.U)
4400
3400
2400
1400
400
Wavenumber (cm-1)

Figure 13: FTIR spectra of UA crystals in the absence and presence of T.ammi extract at pH 4, 4.5, 5

4.8. Powder X-Ray Diffraction (PXRD) Analysis

The PXRD patterns of the three samples, there appears to be no significant change in the crystallinity of uric acid when exposed to the extract. The diffraction peaks for all three conditions without extract, with 1.30 mg/ml extract, and with 2.27 mg/ml extract remain similar in intensity and position. While there are slight variations in peak broadening, these changes are not substantial enough to suggest a major alteration in the crystal structure of uric acid. This implies that the extract, even at higher concentrations, does not cause a significant disruption to the crystallization or overall crystallinity of uric acid under the experimental conditions tested[87].

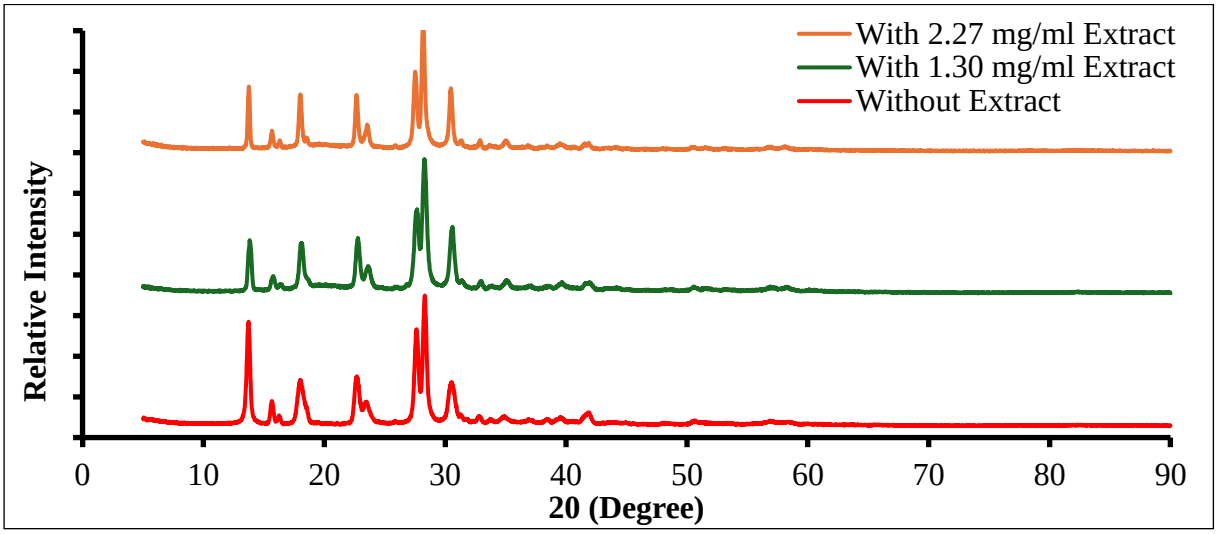

Figure 14: PXRD spectrum of UA crystal in the absence and presence of T.ammi extract at pH 5

4.9. Scanning Electron Microscopy (SEM) and EDS Analysis

The morphological analysis of uric acid kidney stones, before and after treatment with T. ammi extract, was carried out using SEM at 3000× magnification. The untreated sample showed large, well-faceted crystals, indicative of stable uric acid. In contrast, the extract-treated sample displayed disordered and fragmented structures, reflecting reduction in crystal size.

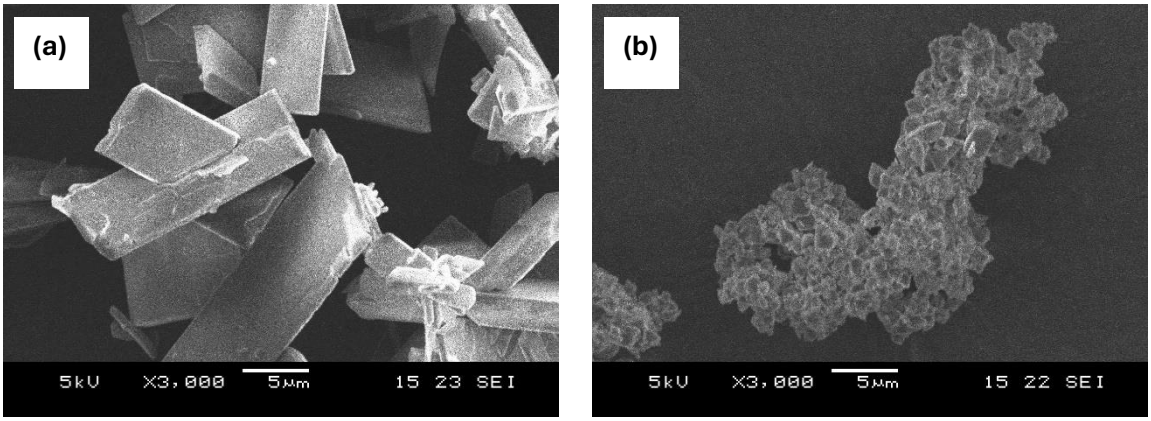

Figure 15: SEM images of UA crystals at pH 5 (a) Without Extract (B) with 2.27 mg/ml Extract

EDS analysis of the untreated sample showed elemental composition as C: 34.22%, N: 33.29%, O: 32.49%. In the treated sample, composition shifted to C: 36.84%, N: 31.13%, O: 32.03%. These variations suggest interaction of phytochemicals from the extract with the uric acid matrix, without causing chemical modifications.

Thus, SEM and EDS confirm that T. ammi extract alters surface morphology of the stone, contributing to its antiurolithiatic potential.

4.10 Dissolution of UA Crystals

UA crystals were synthesized via the method of cooling crystallization. Specifically, 0.1 g of UA was dissolved in 100 ml of distilled water, and the pH was carefully adjusted to 6.0 using dilute solutions of NaOH and HCl. The solution was maintained at a constant agitation speed of 240 RPM in a water-jacketed vessel set at 38°C. The mixture was then incubated at 37°C to facilitate slow evaporation and promote crystal formation. Once visible UA crystals formed, they were carefully collected.

The formed uric acid crystals were subsequently subjected to dissolution studies in the presence of T. ammi extract. Upon exposure to the 2.60 mg/ml of T. ammi extract with

160 ml of synthetic urine and 40 ml of water at pH 5, the crystals began to dissolve, with complete dissolution observed within approximately 6 to 10 minutes.

Figure 16: Synthesized UA Stone

5 CONCLUSION

This study highlights the promising role of *Trachyspermum ammi* (T.ammi) extract as a natural inhibitor for uric acid (UA) crystallization, a key contributor to uric acid kidney stones. Using a combination of phytochemical screening, antioxidant assays, and advanced analytical techniques such as FTIR, PXRD, and SEM-EDS, this thesis demonstrated that T.ammi extract can significantly delay UA crystal formation, reduces crystal size, and alter their morphology without modifying the stones chemically.

The rich presence of bioactive compounds like phenols, flavonoids, and particularly thymol is believed to be responsible for these impacts. These compounds not only provide antioxidant benefits but also appear to interfere with the nucleation and growth of uric acid crystallization. The extract extended induction time under varying pH conditions and concentrations, and microscopy confirmed molecular-level interactions that led to crystal structure disruption and surface modification.

Taken together, these results suggest that T.ammi could serve as a safe, medicinal plant-based alternative for managing or possibly preventing UA stone formation. Its antioxidant-rich profile and its ability to influence crystal growth dynamics support its potential use in herbal medicine and nephrology. Additionally, as a natural product, it offers a potentially cost-effective solution with fewer side effects compared to conventional treatments.

However, this study was conducted using synthetic urine, which does not fully replicate the complexity of real human urine that contains various organic and inorganic constituents. Future research should therefore evaluate the extract's performance in real urine environments to better assess its clinical relevance. It is also important to investigate the bioavailability, metabolism, and pharmacokinetics of the active compounds in T.ammi to understand how they function in the human body.

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